1: Plant Mol Biol. 1992 Jan;18(1):65-78. Related Articles, Links

Activation of a truncated PR-1 promoter by endogenous enhancers in transgenic plants.

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PR-1 genes are induced by various environmental stimuli such as pathogen attack or exposure of the plants to certain chemicals. To examine the regulation of these genes, the 5' flanking regions of the PR-la gene and of two PR-1 pseudogenes were joined by a transcriptional fusion to the Escherichia coli beta-glucuronidase (GUS) gene. These constructs were stably integrated into the tobacco genome and independent primary transformants were monitored for the expression of the reporter gene. Unexpectedly, out of 55 transformants analysed, four plants exhibited considerable GUS activities without any inductive treatment of the plants. Expression of the endogenous PR-1 genes, however, could not be detected in these plants. Primer extension analyses revealed correct initiation of the PR1/GUS hybrid transcripts from the PR-1a TATA box. When the plants were analysed at the cellular level, clear differences regarding the tissue specificity of expression of the reporter gene were observed. These results strongly suggest that the PR1/GUS hybrid promoter expression cassettes may be activated when integrated in the vicinity of heterologous enhancer elements dispersed in the tobacco genome. In order to support this hypothesis, domain B of the enhancer of the 35S RNA promoter from cauliflower mosaic virus (CaMV) was fused to various PR1/GUS hybrid genes upstream as well as downstream from the RNA start site. These constructs were stably introduced into the tobacco genome. In any primary transformant analysed, strong GUS activities were observed with the PR1/GUS hybrid RNAs originating from the normal transcription start site of the PR-1a gene. The tissue specificity of gene expression was identical to that described previously for the CaMV 35S domain B enhancer element. Thus, modulations of the transcriptional activity of the PR-1 promoter can be achieved by heterologous enhancers in transgenic plants and may be encountered upon random integration of PR-1 promoter constructs into the tobacco genome.

PMID: 1731979 [PubMed - indexed for MEDLINE]

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1: Plant J. 1993 Aug;4(2):225-34.

A basic-type PR-1 promoter directs ethylene responsiveness, vascular and abscission zone-specific expression.

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Pathogenesis-related (PR) proteins form a heterogeneous group of host-encoded, low-molecular-mass proteins that are secreted through the exocytic pathway. They are synthesized by the plant in response to various stimuli, including pathogen attack or exposure to certain chemicals. The PRB-1b gene of Nicotiana tabacum codes for a basic-type PR-1 protein whose transcription is regulated by ethylene. A minimal ethylene-responsive promoter element was defined by deletion analysis in transgenic tobacco plants. Promoter sequences containing 213 bp or more were sufficient to enhance a 20-fold increase of beta-glucuronidase reporter gene expression in transgenic tobacco leaves exposed to 20 microliters l-1 of ethylene, while 67 bp were not sufficient to trigger ethylene responsiveness. All the constructs that retained ethylene inducibility exhibited phloem-specific activity, which was constitutive in petiole and pedicel abscission zones. This functional study was correlated to an in vitro screening of the major nuclear proteins' binding sites present on the promoter. Gel-shift analysis using nuclear extracts from ethylene-treated and non-treated plants revealed five sequence-specific protein-DNA complexes on promoter sequences spanning -863 to -142 bp. Constitutive expression of the basic-type PR-1 genes at the leaf and petiole or flower and pedicel interfaces may represent pre-emption of plant defenses against potential pathogens, suggesting a functional similarity to pathogen-induced expression in the leaf.

PMID: 8220480 [PubMed - indexed for MEDLINE]

1: Plant J. 1993 Sep;4(3):481-93.

Pathogen, salicylic acid and developmental dependent expression of a beta-1,3-glucanase/GUS gene fusion in transgenic tobacco plants.

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The 5' flanking region of a gene encoding an acidic beta-1,3-glucanase from Nicotiana tabacum was isolated and characterized. A chimeric gene composed of 1759 bp of the promoter sequence from the PR-2 gene was fused to the beta-glucuronidase (GUS) coding region and used to transform tobacco. Transcriptional activation of the PR-2 promoter was investigated in response to inoculation with tobacco mosaic virus (TMV), after treatment of leaves with salicylic acid (SA), and in specific tissues during the normal development of healthy plants. In TMV-inoculated transgenic plants, GUS activity was induced locally around necrotic viral lesions and systemically in uninoculated leaves. GUS activity was also induced by treatment of leaves with SA. The chimeric gene was expressed in floral organs of healthy plants and in newly germinated seedlings. Analyses of a series of 5' deletions of the glucanase promoter indicated that the cis-acting elements necessary for induction by all these signals are localized in the region between -321 bp and -607 bp upstream of the transcription start site.

PMID: 8220491 [PubMed - indexed for MEDLINE]

1: Plant J. 1996 Dec;10(6):1089-101.

Identification of a salicylic acid-responsive element in the promoter of the tobacco pathogenesis-related beta-1,3-glucanase gene, PR-2d.

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The tobacco pathogenesis-related PR-2d gene encodes an acidic beta-1,3-glucanase. Expression of the PR-2d: uidA(GUS) chimeric gene is induced in leaves undergoing the hypersensitive resistance response to tobacco mosaic virus and after treatment with salicylic acid (SA), a chemical believed to play an important role(s) in disease resistance. We have constructed transgenic tobacco plants which carry various segments of the PR-2d promoter fused to a heterologous core 35S promoter driving the uidA(GUS) reporter gene. Their analysis indicates that sequences from -364 to -288 upstream of the PR-2d transcription start site confer a high level of activation by SA (20-fold). Mutations within this sequence, located between -339 and -333, depressed SA activation. This region is also required for the SA-inducibility of a truncated PR-2d:GUS chimeric gene. Contained within this region is a 25 bp element located between -348 and -324 which was specifically recognized by nuclear factors from tobacco leaves. No conclusive differences were observed in the ability of proteins in nuclear extracts from water-treated versus SA-treated plants to bind to this cis element in vitro.

PMID: 9011089 [PubMed - indexed for MEDLINE]

1: Mol Plant Microbe Interact. 1997 Jan;10(1):69-78. Related Articles, Links

Characterization of a salicylic acid-insensitive mutant (sai1) of Arabidopsis thaliana, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene.

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Salicylic acid (SA) plays an important signaling role in the resistance of many plants to pathogen invasion. Increases in endogenous SA levels have been associated with the hypersensitive response as well as systemic acquired resistance (SAR). SA also induces the expression of a subset of the pathogenesis-related (PR) genes. However, relatively little is known about the events occurring subsequent to SA accumulation during a resistance response. In order to identify mutations in components of the SA signal transduction pathway, we have developed a genetic screen in Arabidopsis thaliana that utilizes the Agrobacterium tumefaciens tms2 gene as a counter-selectable marker. SA-inducible expression of the tms2 gene from the tobacco PR-1a promoter confers sensitivity to alpha-naphthalene acetamide (alpha-NAM), resulting in inhibition of root growth in germinating transgenic Arabidopsis seedlings. Mutants in which root growth is insensitive to alpha-NAM have been selected from this PR-1a:tms2 transgenic line with the expectation that a subset will lack a regulatory component downstream of SA. The sail mutant so identified expressed neither the PR-1a:tms2 transgene nor the endogenous Arabidopsis PR-1, PR-2, and PR-5 genes in response to SA. These genes also were not induced in sai1 by 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole (BTH), two chemical inducers of SAR. As expected of a mutation acting downstream of SA, sai1 plants accumulate SA and its glucoside in response to infection with an avirulent pathogen and are more susceptible to this avirulent pathogen than the wild-type parent. sai1 is allelic to npr1, a previously identified SA-noninducible mutation. The recessive nature of the noninducible sai1 mutation suggests that the wild-type SAI1 gene acts as a positive regulator in the SA signal transduction pathway.

PMID: 9002272 [PubMed - indexed for MEDLINE]

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1: Mol Plant Microbe Interact. 1997 Jul;10(5):624-34. Related Articles, Links

Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development.

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Pathogenesis-related (PR) proteins form a heterogeneous family of plant proteins that are likely to be involved in defense and are inducible by pathogen attacks. One group of PRs, represented by the subfamily PR-1, are low-molecular-weight proteins of unknown biochemical function. Here we describe the cloning and characterization of two closely related genes encoding a basic and an acidic PR-1 protein (PR1b1 and PR1a2) from tomato (Lycopersicon esculentum). We present a comparative study of the mode of transcriptional regulation of these two genes in transgenic tobacco plants using a series of promoter-GUS fusions. Unexpectedly, the chimeric PR1a2/GUS gene is not induced by pathogenic signals but instead shows constitutive expression with a reproducible developmental expression pattern. It is expressed in shoot meristems, trichomes, and cortical cells as well as in vascular and nearby tissues of the mature stem. This constitutive expression pattern may represent preemption of plant defenses against potential pathogens. Conversely, the chimeric PR1b1/GUS gene does not show any constitutive expression in the plant, but it is transcriptionally activated following pathogen attack. Upon infection by tobacco mosaic virus, the PR1b1 gene is strongly activated locally in tissues undergoing the hypersensitive response but not systemically in uninoculated tissues. Furthermore, its expression is induced by both salicylic acid and ethylene precursors, two signals that coexist and apparently mediate the activation of local defenses during the hypersensitive response. We speculate that the different mode of expression of the two genes presented here, together with that reported previously for the induction of other PR-1 genes in systemic, uninoculated tissues, may all be complementary and necessary for the plant to acquire an efficient refractory state to resist pathogen attacks.

PMID: 9204567 [PubMed - indexed for MEDLINE]

1: Plant J. 1993 Feb;3(2):191-201.

Isolation of an asparagus intracellular PR gene (AoPR1) wound-responsive promoter by the inverse polymerase chain reaction and its characterization in transgenic tobacco.

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The Asparagus officinalis intracellular PR1 (AoPR1) gene is expressed in response to wounding and pathogen attack. We utilized the inverse polymerase chain reaction (IPCR) to isolate the cis-acting regulatory sequences of the AoPR1 gene following unsuccessful attempts to identify hybridizing clones in genomic libraries. Sequence analysis of two IPCR products revealed that a 347 bp intron was present in the AoPR1 gene and that it was probable that the AoPR1 regulatory sequence had been amplified. To test the AoPR1 cis-acting sequences for biological function a translational fusion was constructed with the beta-glucuronidase (GUS) reporter gene and tested in tobacco. These data demonstrated that sequences 982 bp from the probable start of transcription are sufficient to direct wound-inducible transcription and that there is no signal peptide encoded by the first 31 residues of the predicted AoPR1 protein. Histochemical localization of GUS activity in transgenic tobacco demonstrated strong activity localized to wound and pathogen invasion sites. GUS activity was also found in mature pollen grains.

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A Mutation in Arabidopsis That Leads to Constitutive Expression of Systemic Acquired Resistance

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Systemic acquired resistance (SAR) is a nonspecific defense response in plants that is associated with an increase in the endogenous level of salicylic acid (SA) and elevated expression of pathogenesis-related (PR) genes. To identify mutants involved in the regulation of PR genes and the onset of SAR, we transformed Arabidopsis with a reporter gene containing the promoter of a β-1,3-glucanase–encoding PR gene (*BGL2*) and the coding region of β-glucuronidase (*GUS*). The resulting transgenic line (*BGL2–GUS*) was mutagenized, and the M_2 progeny were scored for constitutive GUS activity. We report the characterization of one mutant, *cpr1* (constitutive expresser of PR genes), that was identified in this screen and shown by RNA gel blot analysis also to have elevated expression of the endogenous PR genes *BGL2*, PR-1, and PR-5. Genetic analyses indicated that the phenotype conferred by *cpr1* is caused by a single, recessive nuclear mutation and is suppressed in plants producing a bacterial salicylate hydroxylase, which inactivates SA. Furthermore, biochemical analysis showed that the endogenous level of SA is elevated in the mutant. Finally, the *cpr1* plants were found to be resistant to the fungal pathogen *Peronospora parasitica* NOCO2 and the bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326, which are virulent in wild-type *BGL2–GUS* plants. Because the *cpr1* mutation is recessive and associated with an elevated endogenous level of SA, we propose that the *CPR1* gene product acts upstream of SA as a negative regulator of SAR.

INTRODUCTION

Systemic acquired resistance (SAR) is a general defense response that, when activated, protects plants from infection by a wide variety of pathogens (reviewed in Kuc, 1982). The resistance response can be activated by both biological and chemical inducers; biological inducers include avirulent pathogens that usually cause necrotic lesions (Ross, 1961; Hecht and Bateman, 1964; Lovrekovich et al., 1968), whereas chemical inducers include salicylic acid (SA; White, 1979) and 2,6-dichloroisonicotinic acid (INA; Metraux et al., 1991). The appearance of SAR is accompanied by elevated levels of endogenous SA (Malamy et al., 1990; Metraux et al., 1991) and the expression of genes encoding pathogenesis-related (PR) proteins (Van Loon and Van Kammen, 1970; Ward et al., 1991).

A causal link between PR proteins and SAR has yet to be established, but the timing of PR gene expression correlates with the onset and duration of SAR (Ward et al., 1991; Uknes et al., 1992). Furthermore, some of the PR genes encode extracellular proteins with β-1,3-glucanase and chitinase activities. Purified chitinase and β-1,3-glucanase have been shown to inhibit the growth of several fungal pathogens (Schlumbaum et al., 1986; Mauch et al., 1988). β-1,3-Glucanase has also been suggested to be involved in releasing defense-activating elicitors (Keen and Yoshikawa, 1983; Mauch and Staehelin, 1989). Moreover, elevated levels of some of the PR proteins in plants have been shown to confer resistance to certain pathogens. For example, transgenic tobacco plants constitutively producing a chitinase are resistant to the fungal pathogen *Rhizoctonia solani* (Broglie et al., 1991), and plants constitutively expressing the PR-1a gene are resistant to the fungal pathogens *Phytophthora parasitica* and *Peronospora tabacina* (Alexander et al., 1993). Because of these associations with pathogen resistance, PR gene expression is used as a molecular marker for SAR.

Although little is known about the signaling pathway that leads to systemic resistance and the expression of PR genes, the importance of SA as a signal molecule for SAR has been well documented. Treatment of tobacco with exogenous SA induces resistance to pathogens (White, 1979) and expression of PR genes (Ward et al., 1991). Furthermore, increases in endogenous SA levels after infection with an avirulent pathogen have been demonstrated in tobacco, cucumber, and Arabidopsis (Malamy et al., 1990, 1992; Metraux et al., 1990; Rasmussen et al., 1991; Yalpani et al., 1991; Enyedi et al., 1992; Uknes et al., 1993). In addition, a soluble SA binding protein has been identified in tobacco whose binding affinity and specificity

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suggest its involvement in transducing the SA signal (Chen and Klessig, 1991); this SA binding protein has recently been shown to be a catalase whose activity is inhibited by SA (Chen et al., 1993). The most direct evidence that SA plays an essential role in SAR comes from studies of transgenic tobacco plants expressing the salicylate hydroxylase–encoding gene *nahG*, which prevents the accumulation of SA by converting it to catechol. These transgenic plants fail to develop SAR after pathogen infection (Gaffney et al., 1993; Vernooij et al., 1994). However, grafting experiments done between this transgenic line and a wild-type line indicate that, although SA is required in the distal tissues for the establishment of the resistant state, it is not the systemic signal for SAR (Vernooij et al., 1994).

Previous studies have verified that Arabidopsis exhibits the characteristics of SAR, including development of SAR after a hypersensitive response (HR) to an avirulent pathogen (Dempsey et al., 1993; Uknes et al., 1993; Cameron et al., 1994; Mauch-Mani and Slusarenko, 1994), responsiveness to SA and INA induction (Uknes et al., 1992), and expression of PR genes (Uknes et al., 1992; Dempsey et al., 1993). Studies of SAR in Arabidopsis have led to the identification of one class of mutants in which lesions resembling an HR form spontaneously, followed by induction of SAR; these include *acd2* (accelerated cell death; Greenberg et al., 1994), *lsd1*, *lsd2*, *lsd3*, *lsd4*, and *lsd5* (lesions simulating disease; Dietrich et al., 1994). However, mutations affecting signal transduction downstream of the HR have no known morphological phenotype and are thus difficult to identify. To aid in the isolation of such mutants, we constructed a resistance-related reporter gene and used it to transform Arabidopsis; this reporter gene contains the promoter of the *BGL2* gene (a PR gene encoding a β-1,3-glucanase) fused with the coding region of the β-glucuronidase (*GUS*) gene. We have previously reported the use of this reporter gene to isolate a mutant that is a nonexpresser of PR genes in the presence of SA or INA (*npr1*; Cao et al., 1994).

In this paper, we describe the isolation and characterization of an Arabidopsis mutant that does not spontaneously form HR-like lesions and yet constitutively expresses the *BGL2–GUS* reporter gene. Because other, endogenous PR genes are also constitutively expressed in this mutant, we have named it *cpr1* (constitutive expresser of PR genes 1). Significantly, *cpr1* plants are resistant to both the fungal pathogen *Peronospora parasitica* NOCO2 and the bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326 (Davis et al., 1991; Dong et al., 1991), which further indicates that we have identified a mutation leading to constitutive activation of SAR.

RESULTS

Identification of the *cpr1* Mutant Using the *BGL2–GUS* Reporter Gene

At the time we began our studies, there were no morphological phenotypes reported to be associated with mutants in SAR signaling. To circumvent this problem, we transformed Arabidopsis with a reporter gene responsive to resistance-inducing signals. This reporter gene contained the promoter of *BGL2* and the *GUS* coding region. Our screening rationale was that the reporter should reflect the expression of the endogenous PR genes and that in searching for mutants with aberrant expression of the reporter gene, we would identify mutations affecting the regulation of SAR.

There are three β-1,3-glucanase genes in Arabidopsis, *BGL1*, *BGL2*, and *BGL3*, which are in tandem array on a 12-kb segment of chromosomal DNA (Dong et al., 1991). A 2025-bp XbaI-SphI fragment spanning the entire region between *BGL1* and the start codon of *BGL2* was fused to the coding sequence of the *GUS* gene; an EcoRI-SalI fragment containing this chimeric gene was then placed in the transformation vector pBI101 with a selectable marker for kanamycin resistance, as shown in Figure 1. The construct was delivered into Arabidopsis through root transformation (Valvekens et al., 1988). The resulting transformants were tested for kanamycin resistance, and the insertion of *BGL2–GUS* into the Arabidopsis genome was confirmed by DNA gel blot analysis (data not shown).

In the pilot experiment for the mutant screen, 15-day-old wild-type *BGL2–GUS* seedlings were tested for GUS activity using a microtiter plate assay that scored for the conversion of the GUS substrate 4-methylumbelliferyl β-D-glucuronide (MUG) to the fluorescent product 4-methylumbelliferone (4-MU; Jefferson, 1987). As shown in Figure 2, seedlings that had been grown on agar plates with Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) showed little GUS activity without inducers, whereas seedlings of the same age grown on MS medium supplemented with either 0.5 mM SA or 0.1 mM INA expressed significant amounts of GUS activity. For this assay, leaves were placed into microtiter plate wells containing the substrate solution for GUS; we found that the amount of tissue used had little effect on the outcome of the assay and that tissue processing was not necessary.

Mutagenesis was performed in the *BGL2–GUS* transgenic line by exposing seed (~36,000 total) to ethyl methanesulfonate. The mutagenized seeds were sown onto 12 separate flats and allowed to grow, self-fertilize, and set seed. Using the microtiter plate GUS assay procedure, M_2 plants were tested for constitutive expression of *GUS*. Of 13,883 plants screened, 262 *GUS* expressers were identified, transplanted to soil, and allowed to set seed. GUS activity was assayed again in progeny of 147 of these putative mutants to determine whether the phenotype was heritable, and 28 lines again showed GUS activity. To begin characterization of these mutants, the first line that was found to be homozygous for *GUS* expression was chosen for further analysis. We named this mutant *cpr1* (Figure 2).

PR Genes Are Constitutively Expressed in *cpr1* Plants

A quantitative assay for GUS activity was performed to compare the expression of the reporter gene in the *cpr1* plants (*cpr1/cpr1*) with that of the wild-type *BGL2–GUS* transgenic

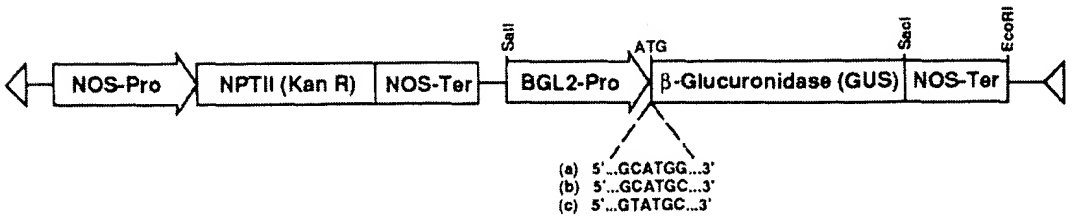

Figure 1. *BGL2–GUS* Reporter Gene Construct.

In (a) are sequences at the translation initiation site of the *BGL2–GUS* reporter gene, a chimera of SphI and NcoI sites; in (b) are sequences at the translation initiation site of *BGL2* after a T-to-C transition at the position −1 to the ATG codon, an SphI site; in (c) are sequences at the translation initiation site of *BGL2*. NOS, nopaline synthase gene; NPTII, neomycin phosphotransferase; Kan R, kanamycin resistance; Pro, promoter; Ter, transcription termination sequence.

line (*CPR1/CPR1*). As shown in Figure 3, the GUS activity observed in the uninduced *cpr1* plants was similar to the level observed in SA- or INA-induced wild-type plants, whereas the level of GUS activity in the uninduced wild-type plants was negligible. Although the addition of SA or INA to *cpr1* plants could further induce GUS activity, the induction was less than twofold. In contrast, the increase in GUS activity observed in the wild-type plants treated with 0.5 mM SA or 0.1 mM INA was 67- and 307-fold, respectively.

Histochemical staining was performed to investigate the pattern of constitutive reporter gene expression in *cpr1* plants and to compare it with that observed in the SA-induced wild type. A consistent pattern was observed in the aseptically grown *cpr1* plants. As shown in Figure 4A, in untreated wild-type seedlings, no GUS activity was observed (the sample on the left), whereas staining was detected in the shoots but not in the roots of untreated *cpr1* seedlings (the sample on the right). More specifically, the stain in *cpr1* plants was present at the tips of young leaves and throughout mature leaves and was concentrated in the veins of old leaves. A similar pattern was observed in the SA-treated wild-type leaves (Figure 4B). Stained tissues were then embedded and sectioned to examine the distribution of GUS activity further. As Figures 4C and 4E indicate, in *cpr1* plants, the reporter gene was expressed mainly in mesophyll cells and vascular tissues, including phloem and young xylem. Strong staining was not found in the epidermis except in stomatal guard cells. This tissue-specific distribution of staining in *cpr1* seedlings was similar to that of the wild type induced by the exogenous application of SA (Figures 4D and 4F).

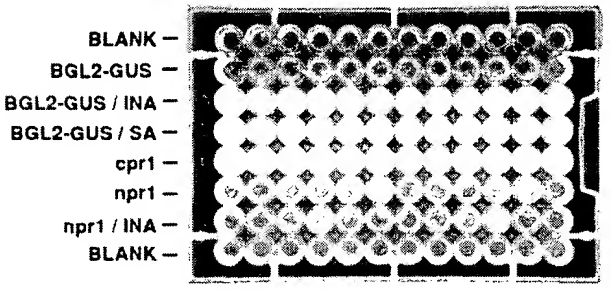

Figure 2. Fluorometric Assay of GUS Activity on a Microtiter Plate as Used for the Mutant Screen.

The plate was illuminated with long-wavelength UV light, which allows detection of the fluorescent product 4-MU formed by hydrolysis of MUG by the GUS enzyme. BLANK indicates that no plant tissue was added. BGL2–GUS, BGL2–GUS/INA, and BGL2–GUS/SA designate wild-type transgenic *BGL2–GUS* plants grown on MS medium, MS medium with 0.1 mM INA, and MS medium with 0.5 mM SA, respectively. cpr1 indicates constitutive expresser of PR genes mutant 1 grown on MS medium; npr1 and npr1/INA designate nonexpresser of PR genes mutant 1 grown on MS medium and MS medium with 0.1 mM INA, respectively.

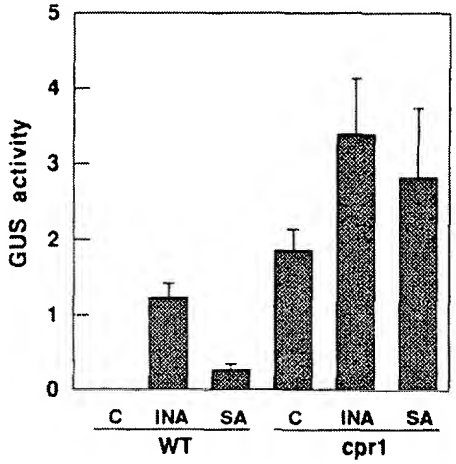

Figure 3. Quantitative Analysis of GUS Activity in Wild-Type and *cpr1* Plants.

Plant tissues assayed were from 15-day-old wild-type (WT) seedlings carrying the *BGL2–GUS* transgene and 15-day-old *cpr1* seedlings. The values represent the average of three replicates ± SE. GUS activity is given as absolute fluorescence units per minute per microgram of protein. C (control) indicates seedlings grown on MS medium; INA, seedlings grown on MS medium with 0.1 mM INA; SA, seedlings grown on MS medium with 0.5 mM SA.

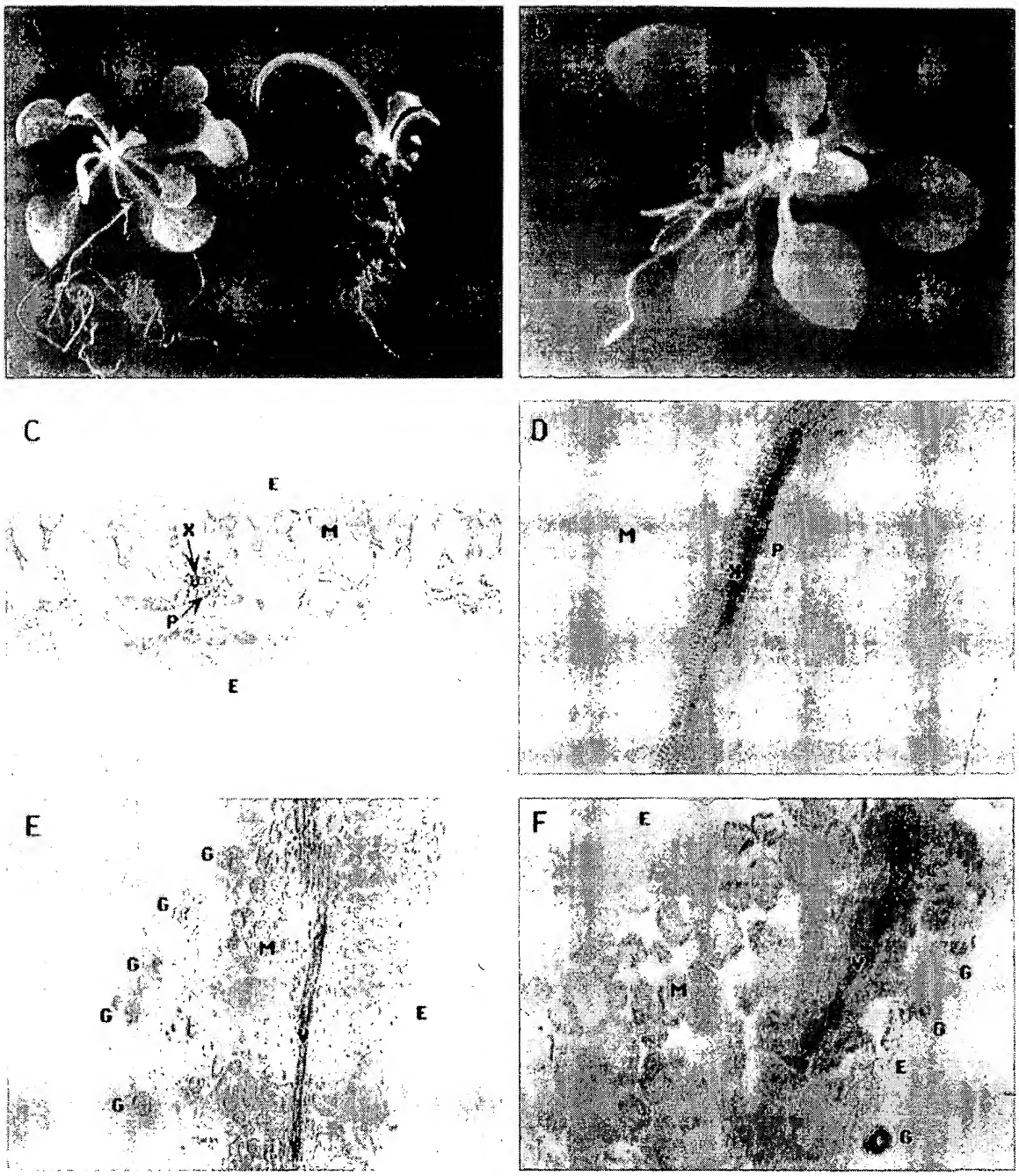

Figure 4. Pattern of *GUS* Expression in *cpr1* Plants Compared with SA-Induced Wild-Type Arabidopsis Plants Containing *BGL2-GUS*.

(A) Histochemical staining of GUS activity in a 15-day-old *cpr1* seedling grown aseptically in MS agar medium (right) and in a wild-type seedling carrying the *BGL2-GUS* transgene grown under the same conditions (left).
(B) An SA-induced *BGL2-GUS* wild-type seedling.
(C) A cross-section of a stained *cpr1* leaf.
(D) A longitudinal section of a stained, SA-induced *BGL2-GUS* wild-type leaf.
(E) A longitudinal section of a stained *cpr1* leaf.
(F) A longitudinal section of a stained, SA-induced *BGL2-GUS* wild-type leaf.
E, epidermis; G, guard cell; M, mesophyll; P, phloem; V, vascular bundle; X, xylem.

To examine the expression of SAR-responsive PR genes, RNA gel blot analyses were performed in both 2-week-old *cpr1* seedlings grown axenically on MS agar medium and 4-week-old mature *cpr1* plants grown on soil. The level of PR mRNA detected in each sample was normalized to that of a constitutively expressed β-ATPase gene and then compared with levels in wild-type plants grown under the same conditions. As shown in Figure 5, levels of the endogenous *BGL2*, PR-1, and PR-5 transcripts were low in wild-type plants, whereas expression of these genes was induced in the presence of 0.1 mM INA. In contrast, the basal level of *BGL2*, PR-1, and PR-5 gene expression was 3.2-, 11.7-, and 1.3-fold higher, respectively, in the *cpr1* seedlings and 4.8-, 12.1-, and 2.6-fold higher in the mature *cpr1* plants compared with the corresponding wild type. Although the amount of PR-5 transcript detected in *cpr1* seedlings was not much more than that in the untreated wild-type seedlings, the induction of PR-5 gene expression produced by INA treatment of the wild-type seedlings was also only 1.3-fold. The presence of 0.1 mM INA in the growth media also led to a further 4.0-fold increase of PR-1 expression in *cpr1* seedlings.

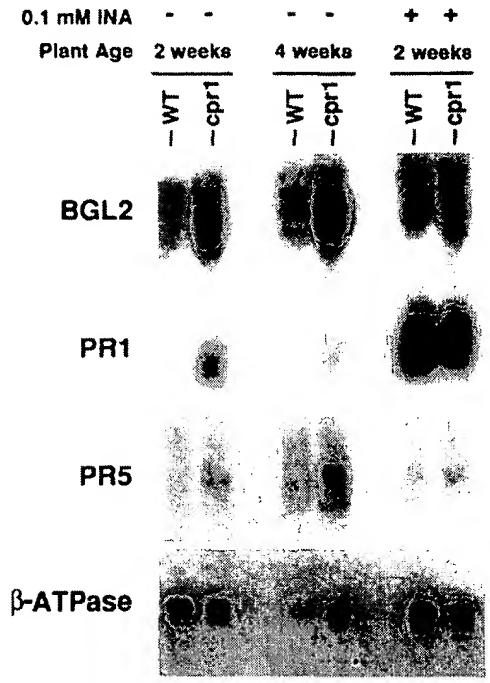

Figure 5. RNA Gel Blot Analysis of *BGL2*, PR-1, and PR-5 in Wild-Type and *cpr1* Plants.

RNA samples were extracted from 2-week-old seedlings grown on MS medium and MS medium with 0.1 mM INA and from 4-week-old plants grown on soil. Endogenous *BGL2*, PR-1, PR-5, and β-ATPase gene–specific probes were used. WT, wild-type *BGL2–GUS* transgenic line; (–) plants grown in the absence and (+) in the presence of INA.

Genetic Analyses Indicate That the *cpr1* Mutation Is Recessive and the *cpr1* Phenotype Is Suppressed by Salicylate Hydroxylase

For genetic analyses, the progeny were assayed for reporter gene expression using both the fluorometric GUS assay in microtiter plates to obtain the ratio of segregation and histochemical staining for GUS activity to verify the accuracy of the fluorometric assay. Plants scored as *cpr1* by the microtiter plate assay were stained and shown always to have the same pattern of staining as the parental *cpr1* plants. Likewise, plants scored as wild type by the microtiter plate assay showed no GUS staining.

To examine the genetic segregation of *cpr1*, a backcross was performed between *cpr1/cpr1* plants and wild-type *CPR1/CPR1* plants containing the *BGL2*–GUS transgene. In the F_1 generation, constitutive expression of *GUS* was absent in all 60 seedlings tested, whereas in the F_2 population, expression was present in 28 of 129 seedlings. The F_2 segregation ratio of the *cpr1* phenotype was 1:3.6, and the chi-square calculated for goodness of fit to a single recessive nuclear mutation was 0.744 ($P > 0.1$).

In addition to constitutive PR gene expression, morphological characteristics were also noted in *cpr1* plants. Compared with the wild type, *cpr1* plants growing on soil were found to have small, narrow, dark green leaves densely covered with trichomes on the adaxial surface and relatively long siliques. Segregation of these visible phenotypes was also studied in the backcross. After scoring for GUS activity, F_2 progeny with high GUS activity (*cpr1/cpr1*) and progeny with no GUS activity (*CPR1/cpr1* and *CPR1/CPR1*) were transplanted to soil, and the morphological phenotypes were examined later. All plants with GUS activity also exhibited the visible phenotypes, whereas all the F_2 progeny without GUS activity looked like the wild type. Therefore, the mutation resulting in the stunted phenotype either is the same mutation that causes the constitutive SAR phenotype or is tightly linked to it.

Further genetic analysis was performed to determine whether the *CPR1* gene product acts upstream or downstream of SA in the signal transduction pathway leading to PR gene expression. We constructed an Arabidopsis transgenic line carrying a bacterial salicylate hydroxylase gene (*nahG*; Yen and Gunsalus, 1982; Schell, 1986; You et al., 1991) controlled by the constitutive cauliflower mosaic virus 35S promoter (see Methods) using a strategy similar to that developed by Gaffney et al. (1993). The expression of the *nahG* gene was confirmed by RNA gel blot analysis, and the salicylate hydroxylase activity in the transgenic plants was shown by the accumulation of a brown oxidation product of salicylate in the roots of the transgenic plants when the growth medium was supplemented with 0.5 mM SA. Crosses were made between homozygous *cpr1* plants and a homozygous transgenic line expressing *nahG*. None of the F_1 progeny constitutively expressed the *GUS* reporter gene; this was expected because *cpr1* is a recessive mutation. To examine whether one copy of the *nahG* gene could produce sufficient levels of salicylate hydroxylase to inactivate SA induction of the reporter gene, F_1 heterozygous

nahG plants were grown in media supplemented with 0.5 mM SA. No reporter gene expression was detected in these plants, indicating that a single copy of the *nahG* gene is sufficient to suppress SA induction. Using this information, two expected ratios of *GUS* expressers to nonexpressers were calculated for the F_2 generation. Due to the involvement of three different loci and selection for kanamycin resistance in our assay, the derivation of these ratios is somewhat complex. The details of the calculation are described in Methods. A ratio of 1:4 would be expected if the *cpr1* phenotype is unaffected by salicylate hydroxylase, meaning *CPR1* acts downstream of SA, whereas a ratio of 1:19 would be expected if salicylate hydroxylase interferes with the *cpr1* phenotype, indicating that *CPR1* functions upstream of SA. In the population of F_2 plants, 28 of 506 plants were positive for GUS activity. This ratio (1:17) argues that salicylate hydroxylase suppresses the *cpr1* phenotype (χ^2 = 0.303; $P > 0.5$) and thus acts upstream of SA. Furthermore, the visible phenotypes associated with *cpr1* plants were mostly suppressed in the F_2 plants that were negative for GUS activity. This implies that these phenotypes are caused by the elevated SA level of *cpr1* plants.

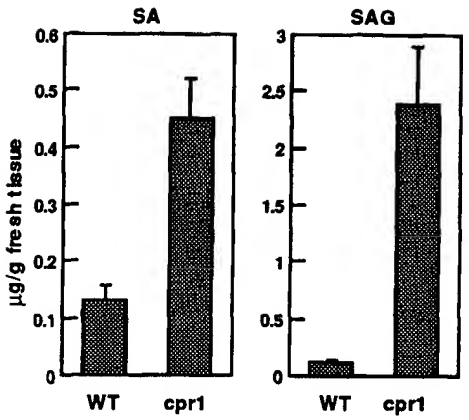

Figure 6. Endogenous Levels of SA and SAG in Wild-Type and *cpr1* Plants.

Leaves from 4-week-old plants grown on soil were harvested and analyzed by HPLC for free SA and SAG content. The values presented are the average of five replicates ± SE. WT, wild-type *BGL2–GUS* transgenic line.

The Endogenous Level of SA Is Elevated in *cpr1* Plants

Because genetic analysis indicated that the *CPR1* gene acts upstream of SA, measurements were made to determine whether the endogenous level of SA was affected in *cpr1* plants. Intracellular SA is found predominantly as free SA or its sugar conjugate, SA β-glucoside (SAG; Enyedi et al., 1992; Malamy et al., 1992). Leaf tissues from 4-week-old *cpr1* and wild-type plants were examined for both free SA and SAG concentrations; the results are presented in Figure 6. In the absence of any inducer, the basal level of SA in *cpr1* plants was 4.5-fold higher than that in wild-type plants, whereas the SAG level was 21-fold higher. The differences in both SA and SAG levels between the wild-type and *cpr1* plants were statistically significant ($P < 0.001$).

cpr1 Plants Are Resistant to the Pathogens *P. parasitica* NOCO2 and *P. s. maculicola* ES4326

To determine whether the elevated levels of SA and PR gene expression in *cpr1* plants lead to the constitutive activation of SAR, the growth of two normally virulent pathogens was examined in *cpr1* plants. To test for resistance to a fungal pathogen, 10-day-old plants were sprayed with a spore suspension of *P. parasitica* NOCO2, which causes downy mildew in Arabidopsis. As shown in Figures 7A and 7B, this pathogen caused disease in the wild-type plants, as indicated by the appearance of many conidiophores on leaves. In contrast, conidiophores were almost completely absent in *cpr1* plants, as shown in Figures 7C and 7D. In a random sample of plants scored 7 days after inoculation, conidiophores were present in 34 of 53 wild-type leaves examined, whereas they were found in only four of 74 *cpr1* leaves scored. To rule out the possibility that the resistance we observed was simply due to the presence of a dense covering of trichomes on *cpr1* leaves that prevented spores from reaching the leaf surface, we examined *cpr1* leaves at a ×4 magnification and confirmed that our method of applying spores using a fine mist thoroughly wets both the adaxial and abaxial leaf surfaces. Furthermore, conidiophores were generally absent even on the abaxial surface of *cpr1* leaves, which are not covered with trichomes. In the wild type, conidiophores were found on both surfaces of the leaves.

Further testing for resistance was performed by infecting 4-week-old wild-type and *cpr1* plants with a bacterial suspension of *P. s. maculicola* ES4326 (see Methods). Plants were examined visually for disease symptoms each day during the week following infection. As shown for plants photographed 4 days after infection (Figure 7E), severe chlorosis was observed in the wild-type leaves 3 days or more after infection, whereas symptoms were nearly absent in *cpr1* plants and in plants treated with INA 2 days prior to infection. The growth of *P. s. maculicola* ES4326 in the plants was also monitored by extracting bacteria from infected leaves at daily time points; the resulting growth curves are shown in Figure 8. The number of *P. s. maculicola* ES4326 detected in *cpr1* plants was more than fourfold lower than that in the wild type after 1 day of growth and nearly sevenfold lower after 2 days. Although the differences between the means of the bacterial concentrations in *cpr1* and wild-type plants were statistically significant at all time points taken after time zero, plants treated with INA had even less bacterial growth. Compared with untreated *cpr1* plants, the INA-treated mutants had fourfold fewer bacteria after 1 day of growth and sixfold fewer after 2 and 3 days.

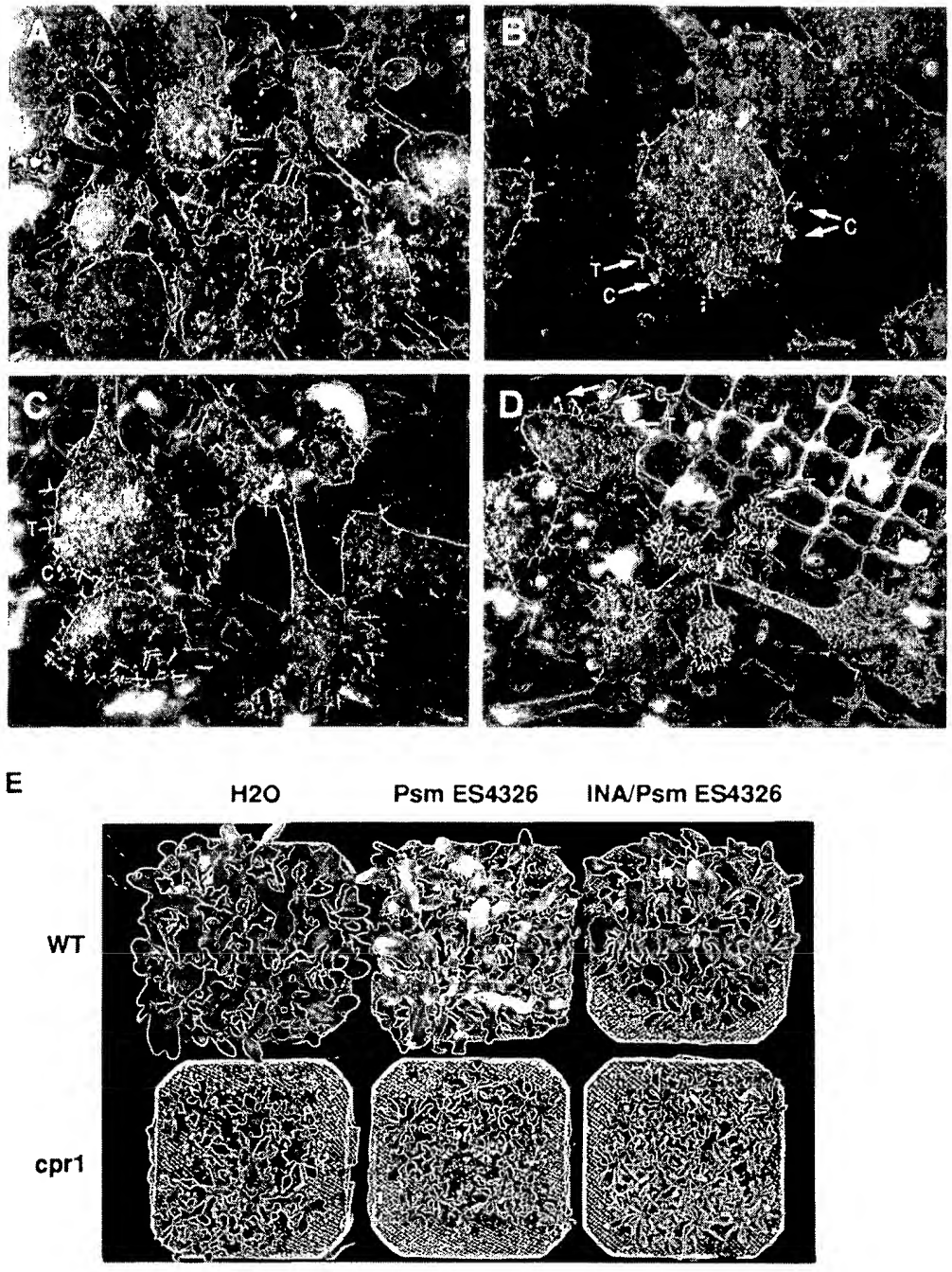

Figure 7. Symptoms Observed in Wild-Type and *cpr1* Plants after Infection with *P. parasitica* NOCO2 and *P. s. maculicola* ES4326.

The *P. parasitica* NOCO2 fungal conidiophore suspension used for infection contained 3 × 10^4 spores per milliliter; the *P. s. maculicola* ES4326 bacteria used for infection were suspended in 10 mM $MgCl_2$, 0.01% surfactant at an OD_{600} reading of 0.2.

(A) and **(B)** Wild-type plants 7 days after inoculation with *P. parasitica* NOCO2.

(C) and **(D)** *cpr1* plants 7 days after inoculation with *P. parasitica* NOCO2.

(E) Wild-type and *cpr1* plants 4 days after being dipped into solutions with or without *P. s. maculicola* ES4326.

C, conidiophore; T, trichome; WT, wild-type *BGL2-GUS* transgenic line; H2O, plants dipped in 10 mM $MgCl_2$, 0.01% surfactant without bacteria; Psm ES4326, plants dipped in the same solution containing 0.2 OD *P. s. maculicola* ES4326; INA/Psm ES4326, plants sprayed with INA 2 days before dipping in the solution containing bacteria.

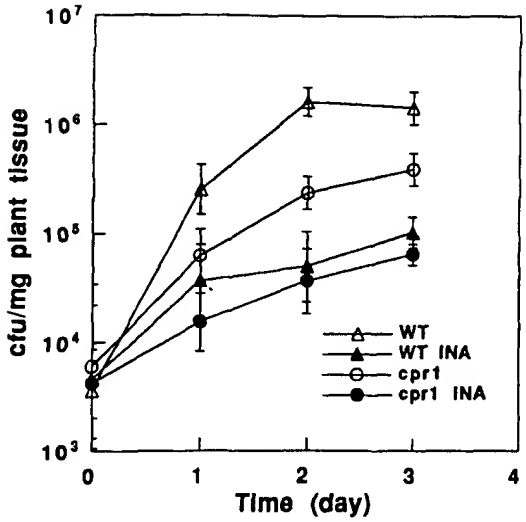

Figure 8. Growth of *P. s. maculicola* ES4326 in Wild-Type and *cpr1* Plants.

Plants were infected by dipping into a *P. s. maculicola* ES4326 bacterial suspension of 10 mM $MgCl_2$, 0.01% surfactant at an OD_{600} reading of 0.2. Samples were taken 0, 1, 2, and 3 days after infection. Error bars represent 95% confidence limits of log-transformed data (Sokal and Rohlf, 1981). Three samples were taken for each genotype at time zero, and thereafter seven samples were taken for each genotype at each time point. cfu, colony-forming units; WT, wild-type *BGL2–GUS* transgenic line; WT INA, wild-type *BGL2–GUS* transgenic line pretreated with INA; cpr1, *cpr1* mutants; cpr1 INA, *cpr1* mutants pretreated with INA.

DISCUSSION

To begin a systematic genetic analysis of the SAR signaling network, we transformed Arabidopsis with a reporter gene (*BGL2–GUS*) responsive to resistance inducers and screened for mutants with aberrant expression of this gene. The assay employed to screen for Arabidopsis mutants had to satisfy the following conditions: (1) the mutants had to be easily identifiable; (2) the assay had to be easily performed; and (3) the mutants had to be rescued to set seed. The GUS assay we derived satisfied these conditions. The combination of the use of microtiter plates and the omission of tissue processing enabled us to screen a large number of mutagenized seedlings and isolate a mutant, *cpr1*, that expressed the reporter gene without the application of exogenous inducers. Our strategy also allowed us to use the *BGL2–GUS* gene to study the levels and tissue-specific pattern of PR gene expression and to perform segregation analysis.

The constitutive expression of *BGL2–GUS* in *cpr1* plants is similar to that found in wild-type plants induced by the chemicals SA and INA. Fluorometric quantitation demonstrated that constitutive GUS activity in *cpr1* plants was at least as high as that in wild-type plants treated with INA and even higher than that in the wild type treated with SA (Figure 3). Also, histochemical staining revealed an expression pattern of the *BGL2–GUS* gene in *cpr1* matching the pattern seen in the chemically induced wild type (Figure 4). This similarity indicates that the constitutive expression of *GUS* in *cpr1* is probably due to a mutation in the same signaling pathway as the one involved in induction after the application of exogenous SA. These common patterns of *BGL2–GUS* expression also imply the presence of a tissue-specific factor(s) regulating PR gene expression.

Besides studies using the reporter gene, RNA gel blot analysis demonstrated that the endogenous PR genes *BGL2*, PR-1, and PR-5 were constitutively expressed in the *cpr1* plants compared with the wild type (Figure 5). This demonstrates that the *BGL2–GUS* reporter gene reflects the expression of endogenous PR genes and that *cpr1* contains a *trans*-acting mutation affecting transcription of these SAR-responsive genes. The presence of other factors that affect the regulation of each specific PR gene, along with such a general regulation modified by the *cpr1* mutation, cannot be ruled out. This is reflected in the RNA gel blot analysis in which differences in the levels of expression of individual PR genes were observed in plants of different ages. Such modifications in patterns of expression may reflect the specific role of each PR protein in SAR.

Because SA appears to be an endogenous signal molecule required for the induction of PR genes (Gaffney et al., 1993), the constitutive expression of PR genes in *cpr1* plants could result from an elevated level of endogenous SA or from the activation of a downstream regulatory factor that transduces the SA signal. The higher level of SA and its glucoside in *cpr1* plants suggests that the constitutive PR gene expression is a consequence of an elevated level of SA (Figure 6). This conclusion is further supported by epistasis studies in which expression of the *nahG* transgene for salicylate hydroxylase, which inactivates SA, suppressed reporter gene expression in *cpr1* plants. Whether the elevated endogenous level of SA in *cpr1* plants is as high as that induced systemically after an HR or as that supplied by exogenous application of SA is not known. Future experiments comparing the SA level in *cpr1* plants with that induced by treatment with avirulent pathogens will address this issue.

Although the molecular characteristics of an SAR response include an increase in the endogenous SA level and the induction of PR genes, the direct physiological evidence of SAR is resistance to a wide range of pathogens. *cpr1* plants that had not been treated with any resistance inducers were challenged with the fungal pathogen *P. parasitica* NOCO2 and the bacterial pathogen *P. s. maculicola* ES4326 and shown to be resistant to both (Figures 7 and 8). Because *cpr1* plants have an elevated level of endogenous SA, constitutively expressed PR genes, and enhanced resistance to very different pathogens, we concluded that *cpr1* is a mutation affecting the SAR signal transduction pathway.

The induction of SAR by the *cpr1* mutation is incomplete. This conclusion is supported by the higher GUS activity detected in the *cpr1* plants treated with either INA or SA (Figure 3), the further induction of the PR-1 gene in INA-treated *cpr1*

seedlings (Figure 5), and the more pronounced reduction of *P. s. maculicola* ES4326 growth in INA-treated *cpr1* plants (Figure 8). This leads us to speculate that the endogenous level of SA in *cpr1* may not be as high as the physiological level normally seen during SAR.

From the phenotype conferred by *cpr1*, we can begin to speculate on the function of the wild-type *CPR1* gene. *CPR1* could be a positively acting factor in the SAR signaling pathway, such as an enzyme involved in the synthesis of SA when activated. In the *cpr1* mutant, a positive factor could be constitutively activated and therefore lead to constitutive expression of SAR. Mutations leading to a constitutive positive signal would most likely be dominant. In contrast, *CPR1* could be a negative regulator in the SAR signaling pathway; it could act as a repressor of either the synthesis or the activity of enzymes involved in SA production. When this repression is inactivated, SAR would be induced. Such a negative factor could be permanently inactivated in the *cpr1* plants, resulting in constitutive SAR. The presence of a wild-type copy of such a gene should override the effect of the mutant copy in heterozygous *CPR1/cpr1* plants; therefore, a mutation in a negative factor would most likely be recessive. Indeed, genetic analysis of progeny from a *cpr1/cpr1* × *CPR1/CPR1* backcross demonstrated that *cpr1* is a recessive mutation. Thus, we propose that the *CPR1* gene functions as a negative regulator acting upstream of SA in the SAR signaling pathway.

SAR is a complex physiological response that can be induced by an HR to a range of viruses, fungi, and bacteria, and it results in enhanced resistance to an even greater number of microbial pathogens. However, the complex network involved in the perception of different pathogens appears to lead to a similar set of responses in the establishment of SAR; thus, a common signaling pathway(s) must exist. Endogenous production of SA appears to be part of such a common pathway (Gaffney et al., 1993). However, several studies suggest that SA is not the systemic signal that is produced at the primary site of infection and translocated throughout the plant to induce SAR (Rasmussen et al., 1991; Vernooij et al., 1994). Rather, SA appears to be required in the distal tissue for response to the unidentified systemic signal. At the end of the signaling pathway, a large number of SAR-related genes, including the PR genes, are induced. This physiological pathway can be overlaid with a genetic pathway, as shown in Figure 9. The previously identified mutations *acd2*, *lsd1*, *lsd2*, *lsd3*, *lsd4*, and *lsd5*, which lead to the spontaneous formation of HR-like lesions accompanied by the induction of SAR, all affect events prior to the establishment of the HR (Dietrich et al., 1994; Greenberg et al., 1994). Such spontaneous necrotic lesions have not been observed in *cpr1* plants; thus, the site of action for *CPR1* seems to be downstream of these HR-regulating genes. However, complementation tests between *cpr1* and *acd2* or *lsd* mutants must be performed to rule out definitively the possibility that *cpr1* is an allele of the known *ACD2* or *LSD* genes that has constitutive SAR in the absence of visible lesions. Furthermore, our studies argue that *CPR1* is a negative regulator acting prior to SA production. Finally, the pathway

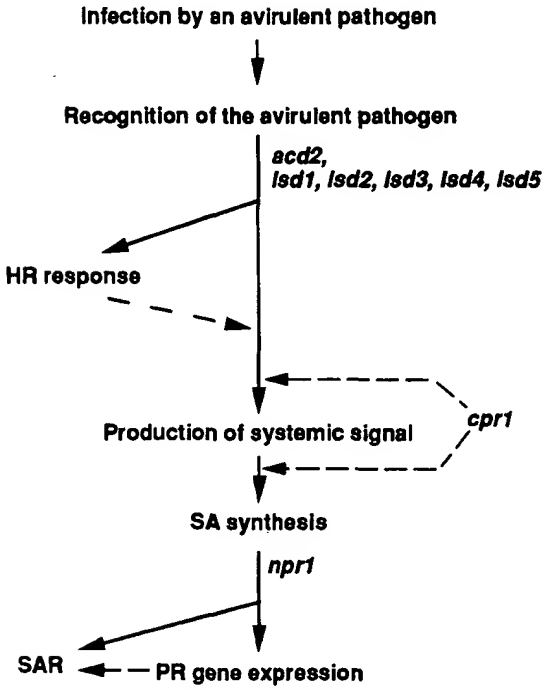

Figure 9. Proposed Placement of Mutants in the SAR Signaling Pathway.

The proposed signal transduction pathway is outlined by solid and dotted lines and arrows. The *cpr1* mutation is placed upstream of SA synthesis, either before or after production of the systemic signal(s).

transducing the SA signal has been marked by another Arabidopsis mutant, designated *npr1*, which is nonresponsive to SA and INA (Cao et al., 1994; Figure 2).

It is interesting that all mutations found thus far that lead to expression of SAR appear to have a detrimental effect on the plants, either by the formation of spontaneous lesions, in the case of *acd2* and *lsd* mutations, or by the appearance of a "stunted" phenotype with *cpr1*. These detrimental effects may explain why SAR is not normally constitutive. The negative effects may offset the advantage of enhanced resistance to pathogens afforded by constitutive SAR.

In summary, we have developed a useful strategy to identify and characterize SAR-related mutants, such as *cpr1*. With these mutants, we can begin genetic analyses to dissect the signaling pathway of SAR. Cloning and further characterization of the regulatory genes associated with these mutants will help to elucidate the molecular basis of SAR.

METHODS

Plant Growth Conditions

Arabidopsis thaliana ecotypes Columbia (Col-0) and Landsberg *erecta* were grown either in pots on Metro-Mix 200 soil (Grace-Sierra, Malpitas,

CA) or on plates with Murashige and Skoog (MS) media (Murashige and Skoog, 1962) containing 2% sucrose and 0.8% agar. Unless otherwise specified, those plants grown on soil were kept in a growth room at high humidity with a 14-hr photoperiod under fluorescent lamps; those grown on MS plates were kept in a growth chamber at 22°C and 70% humidity with a 14-hr photoperiod at a light intensity of 100 μE m^{-2} sec^{-1}. All seeds were vernalized at 4°C for 2 days before placement in a growth environment.

Construction of the Transgenic Line Containing the Reporter Gene

A 2185-bp XbaI-BglII fragment containing the 5′ region of the β-1,3-glucanase (*BGL2*) gene was cloned into the bacterial phage vector M13mp19. To generate an in-frame fusion, an oligonucleotide with the sequence 5′-CCCGTAGCATGCTCCGATTTG-3′ was used to create an SphI restriction site at the initiation codon of *BGL2* by making a T-to-C transition in the position −1 to the ATG codon (Figure 1). The XbaI-SphI fragment was then cloned into the vector pUC19GUS(NcoI) using XbaI-NcoI sites (Dewdney, 1993). The NcoI site in the vector pUC19GUS(NcoI) was at the ATG translation initiation codon of the β-glucuronidase (*GUS*) gene. The SphI-to-NcoI ligation was accomplished by removing the SphI 3′ overhang in the XbaI-SphI fragment by T4 polymerase and filling in the NcoI 5′ overhang of pUC19GUS by using the Klenow fragment of DNA polymerase I (Ausubel et al., 1994). The correct in-frame fusion of the *GUS* coding sequence to the *BGL2* transcription and translation control elements was verified by sequencing. The EcoRI-SalI fragment that contained the *BGL2–GUS* chimeric gene was transferred into the transformation vector pBI101, and the resulting plasmid was used to transform Arabidopsis ecotype Columbia (Valvekens et al., 1988). Transformants were identified by growth in MS medium containing 50 μg/mL kanamycin and by DNA gel blot analysis of the genomic DNA isolated from each transformant (Ausubel et al., 1994).

Mutant Isolation

An Arabidopsis transgenic line carrying the reporter gene *BGL2–GUS* was mutagenized by soaking ~36,000 seeds homozygous for *BGL2–GUS* in 100 mL of 0.3% ethyl methanesulfonate for 11 hr with gentle shaking. Seeds were then washed with water 15 times, resuspended in 0.1% agar, and sown at about 2 per cm^2 of soil. Seventy-five percent of the mutagenized seeds germinated and were allowed to grow, self-fertilize, and set seed. The resulting M_2 seeds were collected in 12 independent pools and germinated on MS agar with 2% sucrose, 50 μg/mL kanamycin, and 100 μg/mL ampicillin. Fifteen days after plating, seedlings were numbered and then a single leaf was removed from each and put into a corresponding microtiter plate well containing 100 μL of GUS substrate solution (50 mM Na_2HPO_4, pH 7.0, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 0.7 μL/mL β-mercaptoethanol, 0.7 mg/mL 4-methylumbelliferyl β-D-glucuronide [MUG]; Jefferson, 1987). Samples were infiltrated with substrate by placing the microtiter plates under vacuum, incubated at 37°C, and then examined for the fluorescent product of GUS activity (4-methylumbelliferone [4-MU]) under a long-wavelength UV light after 3, 6, 12, and 24 hr of incubation. Those M_2 seedlings constitutively expressing GUS activity were considered putative mutants and were transplanted to soil for seed setting. This procedure was repeated with the progeny of these putative mutants to ensure that the mutant phenotype was heritable and to identify the homozygous mutants. Controls included on each microtiter plate were wild-type *BGL2–GUS* plants grown on MS plates alone (for no GUS activity) and grown on MS plates supplemented with either 0.5 mM salicylic acid (SA) or 0.1 mM 2,6-dichloroisonicotinic acid (INA; obtained from Ciba-Geigy LTD, Basel, Switzerland) to induce *GUS* expression.

Quantitative GUS Assay

Fifteen-day-old wild-type and *cpr1* seedlings grown on MS medium, MS medium with 0.5 mM SA, and MS medium with 0.1 mM INA were collected and frozen in liquid nitrogen; three replicates were taken for each genotype and treatment. The samples were homogenized in extraction buffer (50 mM Na_2HPO_4, pH 7.0, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 0.7 μL/mL β-mercaptoethanol; Jefferson, 1987) and centrifuged for 5 min at 14,000 rpm in a microcentrifuge at 4°C. Supernatant aliquots were taken for determination of protein concentration and enzyme activity. The protein concentration was determined using the Bio-Rad dye reagent, and GUS activity was determined using a solution containing 5 μL of extract, 495 μL of extraction buffer, and 500 μL of 2 mM MUG in extraction buffer; the accumulation of the product (4-MU) was measured fluorometrically using a spectrofluorophotometer (RF5000U; Shimadzu Corporation, Kyoto, Japan).

Histochemical Localization of GUS Activity

GUS activity was localized histochemically using a staining solution containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc; first dissolved in dimethyl formamide at 25 mg/mL) in 0.1 M Na_2HPO_4, pH 7.0, 10 mM Na_2EDTA, 0.5 mM potassium ferricyanide/ferrocyanide, 0.06% Triton X-100 (Jefferson et al., 1987). Samples were infiltrated with substrate under vacuum and incubated at 26°C for 24 to 48 hr. The staining buffer was then removed, and the samples were cleared by sequential changes of 30, 75, and 95% ethanol. Sectioning and embedding were performed on some samples, from which slides were prepared for microphotography.

RNA Analysis

Tissue samples were collected from 2-week-old seedlings grown on MS plates or MS plates containing 0.1 mM INA or from 4-week-old plants grown on soil. Samples were frozen in liquid nitrogen after collection, and RNA was isolated from frozen samples by an 80°C phenol–chloroform extraction as previously described (Cao et al., 1994). The RNA concentrations were determined by UV absorbance, and 5-μg samples were separated by electrophoresis through formaldehyde–agarose gels and transferred to a hybridization membrane (GeneScreen; DuPont-New England Nuclear), as described by Ausubel et al. (1994). Ethidium bromide (40 μg/mL) was added to each sample to allow visualization of RNA under UV light for confirmation of equal sample loading. ^{32}P-labeled DNA probes for *BGL2*, pathogenesis-related protein-1 (PR-1), PR-5, and β-ATPase mRNAs were prepared as described by Cao et al. (1994). Hybridization and washing conditions were as previously described (Church and Gilbert, 1984; Cao et al., 1994). Relative radioactivity in each RNA band was determined using a PhosphorImager and ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

Construction of the Transgenic Line Expressing the Salicylate Hydroxylase Gene

A 1.8-kb HindIII-HpaI fragment containing the *nahG* gene encoding salicylate hydroxylase (Yen and Gunsalus, 1982; Schell, 1986; You et al., 1991) was purified from the plasmid pSR20 (M.A. Schell, unpublished data) and cloned into the pBluescript SK+ (Stratagene, La Jolla, CA) HindIII and SmaI sites. Single-stranded DNA was made from this pSKnahG clone using the helper phage R408. A 24-nucleotide oligonucleotide, 5′-ACGAGTACACCATGGAAAACAATA-3′, was used to direct the synthesis of the complementary strand that created a NcoI site at the ATG translation initiation codon of the *nahG* gene. The A-to-G transition in the +4 position of *nahG* also led to a lysine-to-glutamic acid change in the second amino acid of salicylate hydroxylase. The activity of the modified enzyme was tested by plating the bacteria containing this mutant clone on Luria-Bertani medium supplemented with 0.1% SA. Bacteria that produced active salicylate hydroxylase formed brown colonies due to the oxidation of SA by the enzyme. A NcoI-BamHI fragment that contained the entire *nahG* coding sequence was generated by a partial NcoI digestion at the ATG start codon of *nahG* and a complete BamHI digestion in the pBluescript SK+ vector BamHI site. This fragment was then ligated into NcoI and BamHI sites of the vector pRTL2 (Restrepo et al., 1990), which generated an in-frame fusion of the *nahG* gene under the regulation of the following: the cauliflower mosaic virus 35S promoter with a duplicated enhancer; the 5′ untranslated sequence of tobacco etch potyvirus, which enhances translation initiation (Carrington and Freed, 1990; Carrington et al., 1990); and the 35S poly(A) signal. The fusion gene was moved into a Ti plasmid vector pBin19 (Bevan, 1984) through HindIII digestion. This 35S–*nahG* fusion construct was then transformed into Arabidopsis ecotype Landsberg *erecta* (*er/er*) using the transformation procedure previously described by Valvekens et al. (1988).

Genetic Analysis

Crosses were performed by dissecting and emasculating unopened buds and then using the pistils as recipients for pollen from three to four opened flowers. Pollen from homozygous *cpr1* plants was used to fertilize both the wild-type *BGL2–GUS* plants and the transgenic line expressing salicylate hydroxylase (*nahG/nahG*, *er/er*). The reciprocal crosses were also performed. F_1 and F_2 plants were grown on MS plates and tested for GUS activity by the microtiter plate assay and histochemical staining, as previously described. The absence of the recessive *er* phenotype in F_1 plants proved that the crosses with *nahG* plants were successful. GUS activity was scored for F_2 plants grown on MS plates supplemented with 50 μg/mL kanamycin. For the F_2 generation of the *BGL2–GUS* backcross, three-fourths of the plants were expected to have GUS activity if *cpr1* were dominant and one-fourth if *cpr1* were recessive. For analysis of the F_2 generation of the cross with *nahG*, it was known that (1) the *cpr1* mutation is recessive; (2) only one copy of the *BGL2–GUS* gene is required for positive detection of GUS; and (3) a single copy of the *nahG* gene produces a sufficient amount of salicylate hydroxylase to suppress the SA-induced *BGL2–GUS* expression. Thus, the predicted F_2 segregation ratios were calculated as follows: three-fourths of the plants should have at least one copy of the *BGL2–GUS* gene, and one-fourth should have two copies of the recessive *cpr1* mutation; thus, three-sixteenths would be expected to express GUS activity constitutively if *nahG* has no effect on *cpr1*. However, plants were required to have at least one copy of the kanamycin resistance gene carried in either the *BGL2–GUS* or the *nahG* construct to survive the selection. Therefore, one-sixteenth of the plants died, and the expected fraction of *GUS* expressers in the F_2 progeny was modified to three-fifteenths (one-fifth), or a ratio of expressers to nonexpressers of 1:4. The alternative hypothesis that *nahG* suppresses the expression of *BGL2–GUS* in *cpr1/cpr1* plants would predict that among these potential *GUS* expressers, only the one-fourth of them lacking *nahG* should express GUS activity. This brings the predicted fraction of *GUS* expressers in the F_2 generation to one-twentieth (a ratio of 1:19) if the presence of a single *nahG* gene will suppress the *cpr1* phenotype. Segregation in the F_2 generations was analyzed with chi-square tests for goodness of fit (Sokal and Rohlf, 1981).

Determination of Endogenous Levels of SA and SA β-Glucoside

Leaf tissue was collected from 4-week-old wild-type and *cpr1* plants grown on soil, weighed, and frozen in liquid nitrogen. For each sample, ~1 g of the frozen tissue was extracted and quantitated for free SA and SA β-glucoside (SAG) essentially as described previously (Malamy et al., 1992; Hennig et al., 1993). Briefly, the tissue was homogenized in 3 mL of 90% methanol. After centrifugation, the pellet was reextracted with 100% methanol. The combined supernatants were dried in a speed vacuum with heat (~40°C). The residue was resuspended in 2.5 mL of 5% trichloroacetic acid and sonicated for 10 min. The free SA was then separated from conjugated SA through organic extraction with 2 volumes of ethylacetate–cyclopentane–isopropanol (50:50:1). The organic phase containing the free SA was then dried under nitrogen. The dried extract was suspended in 0.5 mL of 0.01 N H_2SO_4, filtered, and analyzed by HPLC as previously described (Hennig et al., 1993). HPLC was performed on an ARH-602 aromatic acids column (0.95 × 10 cm; Interaction Chemical Inc., Mountain View, CA) run at 45°C in 0.01 N H_2SO_4 with a flow rate of 0.6 mL/min.

The amount of SA conjugate was quantitated as follows. After the two-phase separation previously described, the aqueous phase (which contains the conjugated SA) was acidified with HCl to pH 1 and boiled for 30 min to release SA from any acid-labile conjugated forms. The released free SA was then extracted with the organic mixture and analyzed as previously described. To confirm that the SA released from conjugated SA by acid hydrolysis was derived mainly from SAG, we compared SA levels released after either acid hydrolysis or β-glucosidase digestion. Greater than 80% of the acid-releasable SA from Arabidopsis extracts could also be recovered after β-glucosidase treatment. We were unable to use area integration to quantify very low levels of SA reliably (e.g., some of the wild-type samples) because Arabidopsis samples contained large amounts of a fluorescent compound that eluted off the column immediately preceding the SA peak. Despite changing HPLC columns (aromatic acid versus Zorbax phenyl and Zorbax ODS columns; BTR Separations, Wilmington, DE), flow rates, sample volume, and solvent conditions, we have been unable to remove or separate further this unknown contaminant from the SA peak. Therefore, free SA levels, which were often lower than the amounts of the fluorescent contaminant, were determined based on peak height using a standard curve constructed with known amounts of SA. Because this contaminant was extracted into the first organic phase, it did not interfere with determination of the SAG levels, which were quantitated by area integration of the HPLC peaks.

Infection with *Peronospora parasitica* NOCO2

Both wild-type and *cpr1* plants grown on soil with a 12-hr photoperiod were infected with a conidial suspension of *P. parasitica* NOCO2 (3 × 10^4 spores per mL) by spraying and were then kept in a growth chamber at 80% or greater humidity and 18 to 19°C. Seven days after inoculation, plants were scored for the presence of conidiophores using a magnifying glass (×4) and photographed.

Infection with *Pseudomonas syringae* pv *maculicola* ES4326

P. s. maculicola ES4326 was grown at 28°C on King's B agar plates or in King's B liquid medium (King et al., 1954) supplemented with 100 μg/mL streptomycin for selection. Bacteria were then collected by centrifugation and resuspended at OD_{600} = 0.2 in a solution of 10 mM $MgCl_2$ plus the surfactant Silwet L-77 (0.01%; Union Carbide, Danbury, CT). Both wild-type and *cpr1* plants were grown on soil as previously described in 2.5-in square pots covered with fine mesh veil. Four-week-old plants were thoroughly wetted by dipping into the solution described above with or without bacteria. Two days prior to dipping, some plants were sprayed with a 0.65 mM solution of INA. Tissue samples of about 100 mg were collected and weighed at time points of 0, 1, 2, and 3 days after inoculation with bacteria; these samples each contained leaf tissue from 10 to 15 plants growing on one pot. The leaf tissue was homogenized to extract the bacteria as described by Cao et al. (1994). Dilutions were plated on King's B plates with streptomycin for determination of colony-forming units. Statistical analyses were performed by Student's *t* tests of the differences between two means of log-transformed data (Sokal and Rohlf, 1981).

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REFERENCES

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., and Ryals, J. (1993). Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. USA **90**, 7327–7331.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1994). Current Protocols in Molecular Biology. (New York: Wiley Interscience).

Bevan, M.W. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res. **12**, 8711–8721.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Riddle, P., Knowlton, S., Mauvais, C.J., and Broglie, R. (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science **254**, 1194–1197.

Cameron, R.K., Dixon, R.A., and Lamb, C.J. (1994). Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. Plant J. **5**, 715–725.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6**, 1583–1592.

Carrington, J.C., and Freed, D.D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J. Virol. **64**, 1590–1597.

Carrington, J.C., Freed, D.D., and Oh, C.-S. (1990). Expression of potyviral polyproteins in transgenic plants reveals three proteolytic activities required for complete processing. EMBO J. **9**, 1347–1353.

Chen, Z., and Klessig, D. (1991). Identification of a soluble salicylic acid-binding protein that may function in signal transduction in the plant disease-resistance response. Proc. Natl. Acad. Sci. USA **88**, 8179–8183.

Chen, Z., Silva, H., and Klessig, D.F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science **262**, 1883–1886.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**, 1991–1995.

Davis, K.R., Schott, E., and Ausubel, F.M. (1991). Virulence of selected phytopathogenic pseudomonads in *Arabidopsis thaliana*. Mol. Plant-Microbe Interact. **4**, 477–488.

Dempsey, D.A., Wobbe, K.K., and Klessig, D.F. (1993). Resistance and susceptible responses of *Arabidopsis thaliana* to turnip crinkle virus. Phytopathology **83**, 1021–1029.

Dewdney, J. (1993). Light Regulation of Glyceraldehyde-3-Phosphate Dehydrogenase Genes of *Arabidopsis thaliana*. Ph.D. Dissertation (Cambridge, MA: Harvard University).

Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A., and Dangl, J.L. (1994). Arabidopsis mutants simulating disease resistance response. Cell **77**, 565–577.

Dong, X., Mindrinos, M., Davis, K.R., and Ausubel, F.M. (1991). Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell **3**, 61–72.

Enyedi, A.J., Yalpani, N., Silverman, P., and Raskin, I. (1992). Localization, conjugation and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc. Natl. Acad. Sci. USA **89**, 2480–2484.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261**, 754–756.

Greenberg, J.T., Guo, A., Klessig, D.F., and Ausubel, F.M. (1994). Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense functions. Cell **77,** 551–563.

Hecht, E.I., and Bateman, D.F. (1964). Nonspecific acquired resistance to pathogens resulting from localized infections by *Thielaviopsis basicola* or viruses in tobacco leaves. Phytopathology **54,** 523–530.

Hennig, J., Malamy, J., Grynkiewicz, G., Indulski, J., and Klessig, D.F. (1993). Interconversion of the salicylic acid signal and its glucoside in tobacco. Plant J. **4,** 593–600.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusion: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6,** 3901–3907.

Keen, N.T., and Yoshikawa, M. (1983). β-1,3-Endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. Plant Physiol. **71,** 460–465.

King, E.O., Ward, M.K., and Raney, D.E. (1954). Two simple media for the demonstration of phycocyanin and fluorescin. J. Lab. Clin. Med. **44,** 301–307.

Kuc, J. (1982). Induced immunity to plant disease. BioScience **32,** 854–860.

Lovrekovich, L., Lovrekovich, H., and Stahmann, M.A. (1968). Tobacco mosaic virus-induced resistance to *Pseudomonas tabaci* in tobacco. Phytopathology **58,** 1034–1035.

Malamy, J., Carr, J.P., Klessig, D.F., and Raskin, I. (1990). Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science **250,** 1002–1004.

Malamy, J., Hennig, J., and Klessig, D.F. (1992). Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. Plant Cell **4,** 359–366.

Mauch, F., and Staehelin, L.A. (1989). Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell **1,** 447–457.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. **88,** 936–942.

Mauch-Mani B., and Slusarenko, A.J. (1994). Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. Mol. Plant-Microbe Interact. **7,** 378–383.

Metraux, J.-P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science **250,** 1004–1006.

Metraux, J.-P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E. (1991). Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 1, H. Hennecke and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 432–439.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Rasmussen, J.B., Hammerschmidt, R., and Zook, M.N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. Plant Physiol. **97,** 1342–1347.

Restrepo, M.A., Freed, D.D., and Carrington, J.C. (1990). Nuclear transport of plant potyviral proteins. Plant Cell **2,** 987–998.

Ross, A.F. (1961). Systemic acquired resistance induced by localized virus infections in plants. Virology **14,** 340–358.

Schell, M.A. (1986). Homology between nucleotide sequences of promoter regions of *nah* and *sal* operons of NAH7 plasmid of *Pseudomonas putida*. Proc. Natl. Acad. Sci. USA **83,** 369–373.

Schlumbaum, A., Mauch, F., Vögeli, U., and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. Nature **324,** 365–367.

Sokal, R.R., and Rohlf, F.J. (1981). Biometry, 2nd ed. (New York: W.H. Freeman and Company).

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in Arabidopsis. Plant Cell **4,** 645–656.

Uknes, S., Winter, A.M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E., and Ryals, J. (1993). Biological induction of systemic acquired resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. **6,** 692–698.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* root explants using kanamycin selection. Proc. Natl. Acad. Sci. USA **85,** 5536–5540.

Van Loon, L.C., and Van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN.' II. Changes in protein constitution after infection with tobacco mosaic virus. Virology **40,** 199–211.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6,** 959–965.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3,** 1085–1094.

White, R.F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology **99,** 410–412.

Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A., and Raskin, I. (1991). Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell **3,** 809–818.

Yen, K.-M., and Gunsalus, I.C. (1982). Plasmid gene organization: Naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. USA **79,** 874–878.

You, I.-S., Ghosal, D., and Gunsalus, I.C. (1991). Nucleotide sequence analysis of the *Pseudomonas putida* PpG7 salicylate hydroxylase gene (*nahG*) and its 3′-flanking region. Biochemistry **30,** 1635–1641.

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Systemic Acquired Resistance

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INTRODUCTION

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens. After the formation of a necrotic lesion, either as a part of the hypersensitive response (HR) or as a symptom of disease, the SAR pathway is activated. SAR activation results in the development of a broad-spectrum, systemic resistance (Hunt and Ryals, 1996; Neuenschwander et al., 1996). Although SAR is interesting as a paradigm for signal transduction, it may have practical value as well. An understanding of the biochemical changes leading to the resistance state could enable the development of either genetically engineered plants with enhanced disease resistance or novel mode-of-action plant protection chemicals that act by stimulating the plant's inherent disease resistance mechanisms.

SAR can be distinguished from other disease resistance responses by both the spectrum of pathogen protection and the associated changes in gene expression. In tobacco, SAR activation results in a significant reduction of disease symptoms caused by the fungi *Phytophthora parasitica*, *Cercospora nicotianae*, and *Peronospora tabacina*, the viruses tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV), and the bacteria *Pseudomonas syringae* pv *tabaci* and *Erwinia carotovora* (Vernooij et al., 1995). However, the protection is not effective against all pathogens. For example, there is no significant protection against either *Botrytis cinerea* or *Alternaria alternata*. Thus, SAR provides resistance against seven of nine tobacco pathogens, establishing a distinctive fingerprint of protection.

Associated with SAR is the expression of a set of genes called SAR genes (Ward et al., 1991). However, not all defense-related genes are expressed during SAR, and the particular spectrum of gene expression therefore distinguishes the SAR response from other resistance responses in plants. Tobacco is perhaps the best characterized model for SAR, but other plants respond similarly. For example, in Arabidopsis, SAR is effective against *P. parasitica*, *P. syringae* pv *tomato* DC3000, and turnip crinkle virus, and the associated SAR genes are a subset of those expressed in tobacco (Uknes et al., 1992).

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The SAR signal transduction pathway appears to function as a potentiator or modulator of other disease resistance mechanisms. When SAR is activated, a normally compatible plant–pathogen interaction (i.e., one in which disease is the normal outcome) can be converted into an incompatible one (Uknes et al., 1992; Mauch-Mani and Slusarenko, 1996). Conversely, when the SAR pathway is incapacitated, a normally incompatible interaction becomes compatible (Delaney et al., 1994; Mauch-Mani and Slusarenko, 1996). The mechanism by which this modulation occurs is not understood; however, at least part of the resistance response could be due to expression of the SAR genes.

Several comprehensive literature reviews have been published recently (Chen et al., 1995; Hunt and Ryals, 1996; Neuenschwander et al., 1996; Shirasu et al., 1996), so in this article we review recent findings that relate to specific steps in the SAR signal transduction pathway. In particular, we address progress in the identification of biochemical markers for SAR, the role of salicylic acid (SA) in SAR, chemical activators of SAR, and progress in establishing genetic systems to further elucidate steps in the SAR signaling cascade.

MOLECULAR MARKERS FOR SAR

SAR has been recognized as a plant response to pathogen infection for almost 100 years (see Chester, 1933). However, most of the early studies were mainly descriptive and lacked quantitative tools to analyze the response. Thus, considerable effort has been devoted to identifying and isolating biochemical markers for SAR that could be used to distinguish it from other inducible plant resistance responses. A number of biochemical and physiological changes have been associated with pathogen infection. These include cell death and the oxidative burst (Low and Merida, 1996), deposition of callose and lignin (Vance et al., 1980; Kauss, 1987), and the synthesis of phytoalexins (Dixon, 1986) and novel proteins (Bol et al., 1990; Bowles, 1990; Linthorst, 1991; see also Dangl et al., 1996; Hammond-Kosack and Jones, 1996, in this issue). Recently, however, marker genes termed SAR genes have been identified

whose induction is tightly correlated with the onset of SAR in uninfected tissue (Métraux et al., 1989; Ward et al., 1991; Uknes et al., 1992), and these are described in more detail below.

A protein is classified as a SAR protein when its presence or activity correlates tightly with maintenance of the resistance state (Neuenschwander et al., 1996). Analysis of SAR proteins showed that many belong to the class of pathogenesis-related (PR) proteins, which originally were identified as novel proteins accumulating after TMV infection of tobacco leaves (Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970; Van Loon, 1985). In tobacco, the set of SAR markers consists of at least nine families comprising acidic forms of PR-1 (PR-1a, PR-1b, and PR-1c), β-1,3-glucanase (PR-2a, PR-2b, and PR-2c), class II chitinase (PR-3a and PR-3b, also called PR-Q), hevein-like protein (PR-4a and PR-4b), thaumatin-like protein (PR-5a and PR-5b), acidic and basic isoforms of class III chitinase, an extracellular β-1,3-glucanase (PR-Q'), and the basic isoform of PR-1 (Ward et al., 1991). A basic protein family called SAR 8.2 that is induced during the onset of SAR but which shows a pattern of gene expression distinct from that of the other SAR genes has also been described (Ward et al., 1991; Alexander et al., 1992). In Arabidopsis, the SAR marker genes are PR-1, PR-2, and PR-5 (Uknes et al., 1992). The genes encoding these SAR marker proteins have been cloned and characterized and have been used extensively to evaluate the onset of SAR (Ward et al., 1991; Uknes et al., 1992).

Both the expression of marker genes for SAR and the activation of SAR can be triggered by a number of viral, bacterial, and fungal pathogens in a variety of dicotyledonous plants (Neuenschwander et al., 1996); however, the identity and relative expression levels of SAR genes vary between different plant species. For example, in cucumber, acidic PR-1 is weakly expressed (Ryals et al., 1992), whereas in tobacco and Arabidopsis, acidic PR-1 is the predominant SAR-related protein. Such species-specific differences may reflect different evolutionary or breeding constraints that have selected for the most effective SAR response against the particular suite of pathogens to which an individual species is subject (Kessmann et al., 1994).

A number of genes homologous with SAR genes from dicots have been identified in monocot species. Homologs of the PR-1 family have been characterized in maize and barley, and additional PR proteins have been identified in maize (Nassuth and Sanger, 1986; White et al., 1987; Nasser et al., 1988). However, it has not been determined whether the induction of these genes correlates with the onset of SAR in these species. Recently, markers for chemically activated SAR (see below) have been described in wheat (Görlach et al., 1996). These wheat chemically induced (WCI) genes encode a novel lipoxygenase, a cysteine proteinase, and three other proteins whose functions are unknown. The WCI genes are coordinately expressed in response to chemical activators of resistance, and the expression pattern of these genes is similar to those of chemically induced SAR genes in dicot species. However, because a biological model for SAR does not yet exist in wheat, it cannot be confirmed that the WCI genes are bona fide SAR genes (Görlach et al., 1996).

Because the SAR genes are strongly expressed when resistance is maintained, the encoded proteins could contribute to resistance. In support of this idea, in vitro antimicrobial activity has been described for tobacco PR-1a (Sandoz, 1991), chitinases (PR-3; Schlumbaum et al., 1986), β-1,3-glucanases (PR-2), PR-4 (Ponstein et al., 1994), and osmotin (PR-5; Woloshuk et al., 1991). Furthermore, synergistic activity has been found for chitinases and β-1,3-glucanases (Mauch et al., 1988). In vivo studies involving overexpression of PR-1a in tobacco have demonstrated a significant increase in resistance to infection by the two Oomycete pathogens, *P. tabacina* and *P. parasitica* var *nicotianae* (Alexander et al., 1993b). In other experiments, resistance to *P. parasitica* was enhanced in tobacco overexpressing SAR 8.2 (Alexander et al., 1993a), and overexpression of tobacco osmotin partially inhibited growth of *P. infestans* in potato but not in tobacco (Liu et al., 1994). Also, synergistic activity of chitinases and β-1,3-glucanases has been demonstrated in transgenic plants (Zhu et al., 1994; Jach et al., 1995). This evidence suggests that the proteins encoded by the SAR genes are causally associated with disease resistance.

ACCUMULATION OF SA IS REQUIRED FOR SAR SIGNAL TRANSDUCTION

A large body of evidence suggests that SA plays a key role in both SAR signaling and disease resistance. Initially, the level of SA was found to increase by several hundred–fold in tobacco or cucumber after pathogen infection, and this increase was shown to correlate with SAR (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991). Since these reports, a considerable amount of data has established a correlation between the concentration of SA and the establishment of enhanced disease resistance not only in tobacco and cucumber but in other plants as well (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991; Dempsey et al., 1993; Uknes et al., 1993; Yalpani et al., 1993b; Cameron et al., 1994). These data, coupled with the finding that exogenous SA can induce SAR (White, 1979; Ward et al., 1991; Vernooij et al., 1995) and SAR gene expression (Ward et al., 1991; Uknes et al., 1992), led to the suggestion that SA was involved in SAR signaling.

Compelling evidence supporting this idea comes from the analysis of transgenic plants expressing the bacterial *nahG* gene encoding salicylate hydroxylase, an enzyme that catalyzes the conversion of SA to catechol. These plants are not only unable to accumulate free SA, but they are incapable of mounting a SAR response to viral, fungal, or bacterial pathogens (Gaffney et al., 1993; Bi et al., 1995; Friedrich et al., 1995; Lawton et al., 1995), indicating that SA accumulation is required for SAR induction.

Interestingly, in Arabidopsis, depletion of SA causes a breakdown of both SAR and gene-for-gene resistance. Inoculation

of *nahG*-transformed (NahG) Arabidopsis with incompatible races of *P. parasitica* or with strains of *P. syringae* DC3000 carrying an avirulence gene led to the development of severe disease symptoms, which is in contrast to the absence of pathogen growth on isogenic wild-type plants (Delaney et al., 1994). Recently, Mauch-Mani and Slusarenko (1996) used 2-aminoindan-2-phosphonic acid (AIP), an inhibitor of phenylalanine ammonia-lyase (PAL) activity, to block general phenylpropanoid metabolism, which is thought to include the biosynthetic pathway of SA. Pretreatment of Arabidopsis ecotype Col-0 with AIP converts the interaction with *P. parasitica* isolate EMWA from incompatible to compatible. Interestingly, the AIP effect is suppressed by the exogenous application of SA (Mauch-Mani and Slusarenko, 1996). These inhibitor experiments indicate that an important function of PAL activity in plant disease resistance is to provide precursors for the production of SA. Both the phenotype of NahG plants and the AIP experiments suggest that SA is a signal in resistance (*R*) gene–mediated resistance responses and that the ability of the plant to rapidly produce high levels of SA modulates disease resistance.

IS SA THE TRANSLOCATED SIGNAL?

Pathogen infection results in significant amounts of SA in the phloem sap of both cucumber and tobacco (Métraux et al., 1990; Yalpani et al., 1991). Additionally, in vivo SA-labeling studies provide evidence that SA produced in the leaves of TMV-infected tobacco or TNV-infected cucumber is transported throughout the plant and accumulates in uninfected tissues (Shulaev et al., 1995; Mölders et al., 1996). In fact, as much as 70% (tobacco) and 50% (cucumber) of the increase in SA in uninfected tissue of pathogen-inoculated plants results from SA translocation from infected leaves to uninfected leaves.

These data support the contention that SA may be the signal that translocates from an infection site to activate SAR elsewhere in the plant. However, two lines of evidence suggest that SA is not the long distance signal. First, in cucumber, primary leaves infected with *P. syringae* can be removed 6 hr after inoculation, which is before SA accumulates in the phloem, without affecting the systemic increase of SA or SAR gene expression (Rasmussen et al., 1991). Second, in grafted tobacco plants, TMV inoculation of NahG rootstocks resulted in very little SA accumulation in infected tissue, compared with a 185-fold increase for wild-type (Xanthi) plants. However, transmission of the systemic signal out of the NahG rootstocks appeared to be unaffected because the grafted wild-type scions had elevated levels of both SAR gene expression and induced resistance equivalent to those seen in ungrafted wild-type plants (Vernooij et al., 1994).

These results suggest that either SA is not the long distance signal or very small amounts of SA in infected leaves are sufficient for full SAR induction. We have recently found that maximal induction of SAR occurs only at high concentrations of SA in the infected leaf (M.G. Willits and J.A. Ryals, unpublished data). Also, Beffa et al. (1995) have found that transgenic tobacco plants producing cholera toxin form spontaneous lesions, accumulate high levels of SA, and display enhanced disease resistance. In grafting experiments in which these plants are used as rootstocks, the wild-type scions are not induced for SAR, again providing evidence that SA is not the translocated signal.

Even though SA is not likely to be the translocated signal that triggers SAR in distal plant organs, it is essential for SAR signal transduction. Inoculation of wild-type rootstocks with TMV leads to the induction of SAR in wild-type scions but not in NahG scions, demonstrating that the induction of SAR in systemic tissues is SA dependent (Vernooij et al., 1994). These findings indicate that SA is an essential signal in SAR and that it is required downstream of the long distance signal.

BIOSYNTHESIS OF SA

Given the importance of SA in disease resistance, the pathway of SA biosynthesis may represent a major control point in plant defense responses. The biosynthetic pathway of SA appears to begin with the conversion of phenylalanine to *trans*-cinnamic acid (*t*-CA) catalyzed by PAL, as shown in Figure 1. The conversion of *t*-CA into SA has been proposed to proceed via chain shortening to produce benzoic acid (BA), followed by hydroxylation at the C-2 position to derive SA (Yalpani et al., 1993a). The latter step is likely catalyzed by a cytochrome P450 monooxygenase, called benzoic acid 2-hydroxylase (BA2H), the activity of which is induced by either pathogen infection or exogenous BA application (León et al., 1993b). Because exogenous BA causes SA accumulation but *t*-CA does not (Yalpani et al., 1993a), it seems plausible that the rate-limiting step in SA biosynthesis is the conversion of *t*-CA to BA, although other possibilities exist.

The mechanism of BA production from *t*-CA is unknown, but it may occur in a manner similar to the β-oxidation of fatty acids. Evidence for β-oxidation of *t*-CA to BA comes from studies on *Quercus pedunculata*, showing that acetyl-CoA and ATP stimulate the formation of SA from *t*-CA in cell-free extracts (Alibert and Ranjeva, 1971). However, other studies have suggested a nonoxidative mechanism; in suspension cultures of *Lithospermum erythrorhizon* and *Daucus carota*, the conversion of *p*-coumarate (differing from *t*-CA by an additional 4-hydroxyl group) to *p*-hydroxybenzoic acid appears to be independent of acetyl-CoA as a cofactor (Yazaki et al., 1991; Schnitzler et al., 1992). In this case, *p*-hydroxybenzaldehyde is formed as an intermediate, which is also inconsistent with β-oxidation. Both possibilities are illustrated in Figure 1.

Interestingly, both BA and SA can be conjugated to glucose, and regulation of SA levels through SA or BA conjugation may be important. In healthy tobacco plants, there is a large pool of conjugated BA that decreases transiently in size after pathogen infection. The decrease in conjugated BA levels correlates

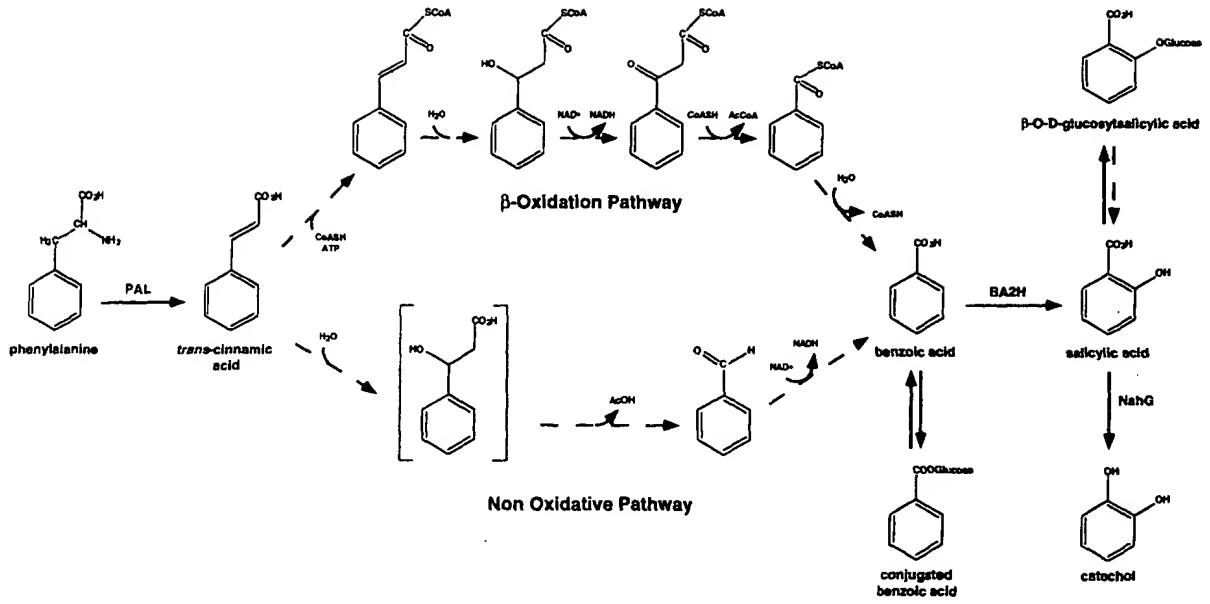

Figure 1. Proposed SA Biosynthetic Pathways in Plants.

Oxidative and nonoxidative pathways for the conversion of *t*-CA to BA, leading to formation of SA, are shown. Solid arrows indicate established biochemical reactions, whereas broken arrows indicate possible steps not yet described (see Yazaki et al., 1991).

with an increase in free BA and SA (Yalpani et al., 1993a). Once SA accumulates, it is rapidly converted to β-O-D-glucosylsalicylic acid (SAG), which apparently is not active in disease resistance (León et al., 1993a). Conversion of SAG to free SA represents another potential mechanism for increasing levels of free SA (Figure 1). In summary, whereas SA appears to play an important role in both SAR and *R* gene–mediated resistance, little is known about its synthesis and degradation.

MODES OF ACTION OF SA

The mechanism by which SA induces SAR is unknown; however, it has been proposed that H_2O_2 acts as a second messenger of SA in SAR signaling. An SA binding protein was identified as catalase; SA was found to inhibit the catalase activity of this protein, leading to elevated levels of H_2O_2. Furthermore, H_2O_2 was found to cause induction of PR-1 gene expression and was postulated to induce SAR (Chen et al., 1993, 1995).

Recent reports, however, indicate that H_2O_2 is not a second messenger of SA in SAR signaling (Bi et al., 1995; León et al., 1995; Neuenschwander et al., 1995b; Summermatter et al., 1995). For H_2O_2 to function as a signaling agent of SA, H_2O_2 levels should increase in uninfected leaves of tobacco plants during SAR activation. This was tested by inoculating leaves of tobacco with TMV and monitoring the accumulation of H_2O_2, PR-1 mRNA, and the establishment of SAR. In the uninfected leaves of inoculated plants, SAR gene expression and the establishment of SAR did not correlate with an increase in H_2O_2 levels. In addition, induction of PR-1 expression by H_2O_2 was directly tested by infiltration of tobacco with H_2O_2. Substantial PR-1 mRNA accumulation resulted after infiltration of 1 M H_2O_2, a concentration that also caused severe tissue damage. However, in NahG plants, 1 M H_2O_2 did not induce PR-1 significantly, indicating a requirement for SA in H_2O_2-mediated PR-1 expression (Neuenschwander et al., 1995b). In fact, high concentrations of H_2O_2 were found to induce SA synthesis in both tobacco (León et al., 1995; Neuenschwander et al., 1995a) and Arabidopsis (Summermatter et al., 1995), suggesting that H_2O_2 may induce PR-1 accumulation through induction of SA. Taken together, these results indicate that H_2O_2 is not a second messenger of SA in the signal cascade leading to establishment of SAR.

If H_2O_2 is not directly involved in SAR signaling, what is the biological significance of the inhibition of catalase by SA? One possibility is that this finding is of little relevance for plant–pathogen interactions. For example, recent reports suggest that very high levels of SA (1 mM) inhibit the in vitro activity of a variety of heme-iron–containing enzymes, including catalase (Chen et al., 1993), ascorbate peroxidase (Durner and Klessig, 1995), and the mitochondrial enzyme aconitase, possibly as a consequence of the reported ability of SA to chelate iron (Rueffer et al., 1995). This effect of SA on enzyme inhibition may have no real biological significance. Alternatively, the inhibition of

catalase and peroxidase could be very important for lesion formation. The K_d for SA binding to catalase and ascorbate peroxidase was reported as 14 μM (Chen and Klessig, 1991) and 78 μM (Durner and Klessig, 1995), respectively. This concentration of SA occurs immediately adjacent to a pathogen-induced lesion but not in uninfected leaves in which SA concentrations are 10- to 100-fold lower (Enyedi et al., 1992; Neuenschwander et al., 1995b). Therefore, the biological significance of SA-mediated inhibition of oxidoreductases may be restricted to local responses in infected tissue.

Interestingly, in parsley cell cultures and cucumber cotyledons, SA pretreatment was recently found to increase dramatically the competence of the tissue to trigger a burst of H_2O_2 in response to subsequent elicitor treatment. This conditioning of cells by SA was dependent on protein synthesis and correlated with enhanced resistance of cucumber cotyledons to the fungal pathogen *Colletotrichum lagenarium*. Moreover, the increase in H_2O_2 levels was not due to a decrease in the rate of degradation but to an increase in H_2O_2 synthesis (Kauss and Jeblick, 1995; Fauth et al., 1996).

Thus, the data now available suggest the existence of more than one mode of action of SA in resistance responses. In uninfected leaves, a high-affinity receptor for SA could mediate the induction of SAR gene expression. After establishment of SAR, the tissue becomes competent for rapid elicitation of an oxidative burst at the site of pathogen attack, as opposed to a slower response in tissues in which SAR has not been established. In infected leaves, high concentrations of SA around the site of infection may inhibit catalase and other oxidoreductases. Inhibition of catalase activity could prolong the half-life of H_2O_2 and lead to an amplification of the oxidative burst. The oxidative burst may trigger a variety of local defense responses (Mehdy, 1994; Levine et al., 1996; see also Hammond-Kosack and Jones, 1996, in this issue), including programmed cell death during the HR as well as defense gene expression and synthesis of SA in adjacent cells. This would create a runaway cycle leading to high levels of both SA and H_2O_2 at the site of pathogen attack. In this model, inhibition of oxidoreductases represents a low-affinity perception mechanism that transduces high local SA levels into local defense responses.

CHEMICAL ACTIVATORS OF SAR

SAR was first described as a response to pathogen infection. Subsequently, it has been found that treatment of plants with low molecular weight molecules can also induce SAR. The use of chemicals to activate SAR provides novel alternatives for disease control in agronomic systems as well as tools for the elucidation of the SAR signal transduction cascade (Neuenschwander et al., 1995a). To be considered an activator of SAR, a chemical should exhibit three characteristics (Kessmann et al., 1994): first, the compound or its significant metabolites should not exhibit direct antimicrobial activity; second, it should induce resistance against the same spectrum of pathogens as in biologically activated SAR; and third, it should induce the expression of the same marker genes as evident in pathogen-activated SAR.

Several chemicals or extracts, including silicon, phosphate, 2-thiouracil, polyacrylic acid, nucleic acids, and fosethyl-Al, have been reported as potential activators of resistance but have failed to fulfill the above criteria (Kessmann et al., 1994). Other compounds, such as DL-3-aminobutanoic acid or probenazole, have been shown to slightly induce either PR-1 gene expression or resistance against one or two pathogens, but activation of bona fide SAR has not been demonstrated (Iwata et al., 1980; Asselin et al., 1985; Cohen et al., 1993).

To date, SA is the only plant-derived substance that has been demonstrated to be an inducer of SAR (White, 1979; Antoniw and White, 1980; Ward et al., 1991). The chemicals 2,6-dichloroisonicotinic acid and its methyl ester (both referred to as INA) were the first synthetic compounds shown to activate SAR, thus providing broad-spectrum disease resistance (Métraux et al., 1991; Vernooij et al., 1995). However, both SA and INA were insufficiently tolerated by some crop plants to warrant practical use as plant protection compounds. Recently, the synthetic chemical benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) was demonstrated to be a potent SAR activator (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996) that supplies protection in the field against a broad spectrum of diseases in a variety of crops. Thus, BTH is an attractive compound for practical agronomic use. The resistance observed in the plants after treatment with INA or BTH is not due to a direct action of the compounds on the pathogen, because neither the compounds nor their significant metabolites exhibit in vitro antibiotic activity (Métraux et al., 1991; Friedrich et al., 1996). Moreover, in tobacco, Arabidopsis, and wheat, INA and BTH induce the same set of SAR genes that is induced by SA (Ward et al., 1991; Uknes et al., 1992; Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996).

Neither INA nor BTH treatment causes elevated levels of SA in the plant, and both compounds activate SAR when applied to NahG plants, suggesting that both INA and BTH act independently or downstream of SA in SAR signaling (Vernooij et al., 1995; Friedrich et al., 1996). INA, BTH, and SA are unable to activate SAR gene expression in the *nim1* mutant (noninducible immunity) of Arabidopsis (see below), suggesting that all three compounds activate the SAR signal transduction pathway through the same signaling cascade (Delaney et al., 1995; Lawton et al., 1996). Furthermore, the structural similarities of the three compounds (Görlach et al., 1996) suggest that they may all bind to the same receptor, although direct evidence for this is lacking.

CONSTITUTIVE SAR MUTANTS

In an effort to identify steps in the SAR signal transduction pathway, several groups have taken a genetic approach. Arabidopsis was chosen as the model plant because it is an established plant system for mutant analysis (Redei and Koncz, 1992) and gene isolation (Meyerowitz, 1992) as well as a facile

system for studying plant–pathogen interactions in general (Dangl, 1993; Kunkel, 1996) and SAR in particular (Uknes et al., 1992; Cameron et al., 1994; Mauch-Mani and Slusarenko, 1994). Mutants that are constitutively activated for SAR or compromised in their ability to launch the SAR response have been identified. Employing these mutants in epistatic analyses is a first step toward elucidating the steps in the pathway leading to SAR activation. A reference compilation of SAR mutants identified to date is provided in Table 1, and a hypothetical schematic of the SAR signal transduction pathway is shown in Figure 2.

SAR is activated by pathogens that trigger or cause a cell death response in plants. The cell death can extend from an HR to disease-related necrosis, but the SAR-specific elements of the death process are not clear. One approach toward understanding the relationship between cell death and SAR is to study mutants that spontaneously exhibit cell death in the absence of pathogens (Langford, 1948; Walbot et al., 1983; Wolter et al., 1993; see also Dangl et al., 1996, in this issue). The identification of these mutants suggests the action of a genetically controlled programmed cell death process. In Arabidopsis, seven mutants called lesions simulating disease (*lsd1* to *lsd7*) and one called accelerated cell death (*acd2*) have been described. In addition to their spontaneous lesion formation phenotype, these mutants display elevated SAR gene expression, high concentrations of SA (see Table 1), and resistance to virulent pathogens (Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al., 1995). These data show that there is a clear link between cell death and SAR induction.

In an effort to determine the epistatic relationship between SA accumulation and cell death in SAR signal transduction, *lsd1*, *lsd2*, *lsd4*, *lsd6*, and *lsd7* were crossed to NahG plants (Weymann et al., 1995; M.D. Hunt, T. Delaney, R. Dietrich, K. Weymann, J. Dangl, and J.A. Ryals, unpublished results; U.H. Neuenschwander, R. Dietrich, J. Dangl, and J.A. Ryals, unpublished results). Consistent with previous reports in which *nahG* expression suppressed SAR gene expression (Delaney et al., 1994; Lawton et al., 1995), progeny of crosses between *lsd2*, *lsd4*, *lsd6*, or *lsd7* and NahG plants did not exhibit PR-1 gene expression. Furthermore, the resistance to a virulent *P. parasitica* isolate evident in the parental mutants was not retained in the progeny of *lsd*/NahG crosses. This is consistent with the lack of SAR activation by necrogenic pathogens that is evident in wild-type plants expressing *nahG* (Gaffney et al., 1993; Delaney et al., 1994; Lawton et al., 1995).

The placement of the lesion mimic mutants in the SAR signal transduction pathway model (Figure 2) was also facilitated by the *nahG* epistasis experiments. Lesion formation remained unchanged for *lsd2* or *lsd4* expressing *nahG* compared with the *lsd2* and *lsd4* parents (M.D. Hunt, T. Delaney, R. Dietrich, K. Weymann, J. Dangl, and J.A. Ryals, unpublished results), indicating that *lsd2* and *lsd4* exhibit cell death that is independent of SA or SA-dependent processes. In contrast, lesion formation was suppressed in *lsd1*, *lsd6*, or *lsd7* mutants ex-

Table 1. Arabidopsis SAR Mutants

Mutant	Ecotype	Mutagen	Chromosome—Dominancy/Recessivity	SA Levels	Comments	References
SAR constitutive						
acd2-2	Col-0	EMS[a]	4—recessive	High	Spontaneous lesions	Greenberg et al. (1994)
cim2	Col-0	EMS	ND[b]—dominant	High	No spontaneous lesions	Lawton et al. (1993)
cim3	Col-0	EMS	ND—dominant	High	No spontaneous lesions	H.-Y. Steiner and J.A. Ryals, unpublished data
cpr1	Col-0	EMS	ND—recessive	High	Lesioned status undetermined	Bowling et al. (1994)
lsd1	Ws-0	T-DNA	4—recessive	High	Spontaneous lesions	Dietrich et al. (1994)
lsd2	Col-0	EMS	ND—dominant	High	Spontaneous lesions	Dietrich et al. (1994)
lsd3	Ws-0	T-DNA	ND—recessive	High	Spontaneous lesions	Dietrich et al. (1994)
lsd4	Ws-0	T-DNA	ND—dominant	High	Spontaneous lesions	Dietrich et al. (1994)
lsd5	Ws-0	T-DNA	ND—recessive	High	Spontaneous lesions	Dietrich et al. (1994)
lsd6	Col-0	EMS	1—dominant	High	Spontaneous lesions	Weymann et al. (1995)
lsd7	Col-0	EMS	ND—dominant	High	Spontaneous lesions	Weymann et al. (1995)
SAR compromised						
NahG	Col-0	NahG	4—dominant	Low	INA rescuable	Lawton et al. (1995)
ndr1	Col-0	Fast neutron	3—recessive	ND	INA rescuable	Century et al. (1995)
nim1	Ws-0	T-DNA	1—recessive	Normal	INA/SA insensitive	Delaney et al. (1995)
npr1	Col-0	EMS	ND—recessive	ND	INA/SA insensitive	Cao et al. (1994)

[a] EMS, ethyl methanesulfonate.
[b] ND, not determined.

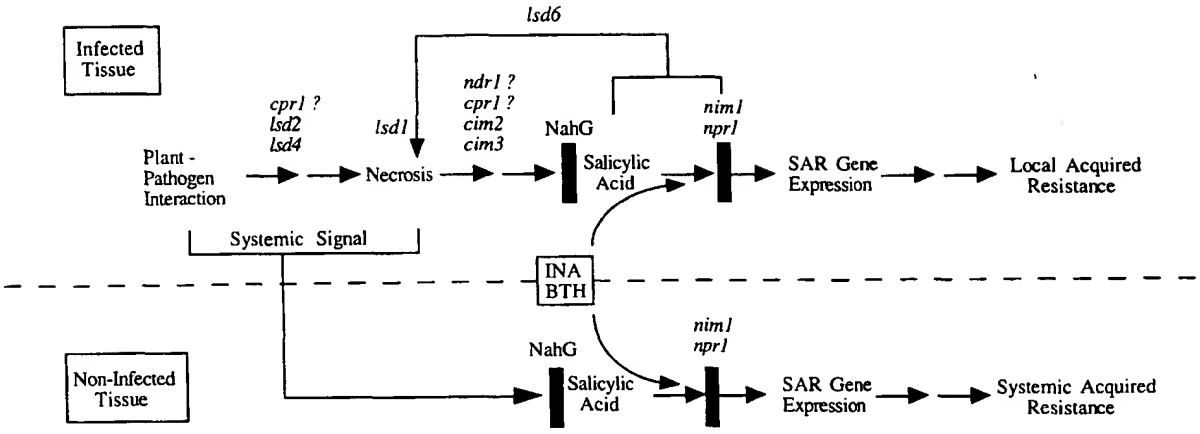

Figure 2. Signal Transduction in SAR.

Pathogen-induced necrosis triggers the activation of both systemic (bottom) and local (top) acquired resistance. Both signaling cascades are SA dependent and are blocked in NahG plants that are unable to accumulate SA due to the expression of salicylate hydroxylase. Lesion formation in the mutants *lsd1* and *lsd6* is SA dependent, suggesting a feedback loop. NahG plants but not the *nim1* and *npr1* mutants can be "cured" by the two activators INA and BTH. ?, hypothetical placings of mutants in the scheme. Adapted with permission from Hunt and Ryals (1996).

pressing *nahG* (U.H. Neuenschwander, R. Dietrich, J. Dangl, and J.A. Ryals, unpublished results; Weymann et al., 1995), indicating that SA or some SA-dependent process is necessary for spontaneous cell death in these mutants.

The results obtained from the crosses of *lsd1*, *lsd2*, *lsd4*, *lsd6*, or *lsd7* with NahG plants are conflicting because they suggest that lesion formation may be positioned both before and after SA accumulation in the signal transduction pathway. Nevertheless, considerable evidence places lesion formation before SA accumulation. For example, the exogenous application of SA to wild-type plants at levels that effectively activate SAR does not induce lesion formation, although at very high concentrations, SA can cause phytotoxicity (Ward et al., 1991). Furthermore, lesion formation in NahG tobacco and NahG Arabidopsis is not inhibited, even though SA levels are markedly reduced (Gaffney et al., 1993; Delaney et al., 1994; Friedrich et al., 1995). One possible explanation for the suppression of lesion formation in the progeny of *lsd1*, *lsd6*, or *lsd7* and NahG crosses is feedback regulation of lesion formation by SA or SA-dependent events. In support of this hypothesis, the *nahG*-expressing *lsd1* or *lsd6* mutants have been shown to regain lesions after INA treatment, indicating that SA-dependent signaling events may indeed regulate processes such as lesion formation (Weymann et al., 1995). The ability to restore lesions by INA application to progeny of *lsd1* or *lsd6* and NahG crosses is compelling evidence for a feedback loop in the SAR signal transduction pathway (see Figure 2).

Arabidopsis mutants also have been identified that possess elevated SA levels and constitutive SAR gene expression in the absence of cell death. Mutants that lack spontaneous cell death but exhibit constitutive SAR are named *cim*, because they exhibit constitutive immunity that renders them resistant to virulent pathogens (Lawton et al., 1993). *cim3* displays no visible or microscopic lesions after trypan blue staining, possesses elevated levels of both free and conjugated forms of SA, and shows constitutive expression of the SAR biochemical markers PR-1, PR-2, and PR-5 (H.-Y. Steiner, S. Uknes, E. Ward, K. Weymann, D. Chandler, S. Potter, and J.A. Ryals, unpublished data). Moreover, *cim3* exhibits heightened resistance to infection with virulent bacterial (*P. syringae* DC3000) and fungal (*P. parasitica*) pathogens. Consistent with previous mutant analyses, inhibition of SA accumulation in *cim3* by expression of *nahG* suppresses both SAR gene expression and resistance to *P. parasitica*. The phenotype of *cim3* indicates that it may encode a gene product that functions at an early step in the signal transduction cascade leading to SAR activation, occurring after cell death but before SA accumulation. Interestingly, Bowling et al. (1994) described and characterized an Arabidopsis mutant that is a constitutive expresser of PR genes (*cpr1*) and that exhibits SAR. However, histochemical staining of leaves to identify the presence of microscopic lesions was not reported, so the classification of this mutant with respect to lesion formation is uncertain.

SAR-COMPROMISED MUTANTS

In addition to mutants with enhanced disease resistance, Arabidopsis mutants that are compromised in their pathogen defense responses have been identified and characterized. Delaney et al. (1995) identified and characterized six allelic recessive mutants named *nim* that are not responsive to

exogenous application of SA or synthetic SAR activators such as INA. Similarly, Cao et al. (1994) have also isolated and described a recessive Arabidopsis mutant called *npr1* (nonexpresser of PR genes), which exhibits compromised activation of SAR. *npr1* may be allelic to *nim1*.

The phenotypes of *nim1* and *npr1* indicate that their block in SAR signaling occurs before SAR gene expression but subsequent to SA accumulation. Evidence for this placement was obtained by analysis of *nim1* plants infected with the avirulent pathogen *P. syringae* DC3000 harboring the cloned *avrRpt2* gene (Whalen et al., 1991). *nim1* plants were shown to accumulate both free and glucose-conjugated SA levels in excess of those in wild-type plants. Therefore, *nim1* plants are able to accumulate SA in response to pathogen infection but appear to be defective in SA perception or in subsequent SA-sensing events. That the *nim1* and *npr1* mutations define genes that act before SAR gene expression was substantiated by the lack of PR-1 induction evident in these plants after INA or SA treatment. Furthermore, *nim1* plants showed reduced and delayed levels of PR-1 mRNA accumulation after infection with the virulent pathogen *P. parasitica* isolate EMWA. Taken together, these results indicate that the *nim1* and *npr1* mutants are compromised in both pathogen-associated and chemical activation of SAR.

In contrast to *nim1* and *npr1*, Century et al. (1995) identified a partial resistance-compromised mutant *ndr1-1* (non-race-specific disease resistance) that is susceptible to several *P. parasitica* isolates (virulent and avirulent) as well as to *P. syringae* DC3000 carrying any one of the cloned bacterial avirulence genes *avrB*, *avrRpm1*, *avrRpt2*, and *avrPph3*. Interestingly, despite its susceptibility to normally avirulent pathovars, the HR of the *ndr1-1* mutant was not substantially different from that in the wild type in response to infection with *P. syringae* harboring *avrB*, *avrRpm1*, or *avrPph3*. However, an HR was not evident in *ndr1-1* after infection with *P. syringae* harboring *avrRpt2*. These results are particularly interesting because of the apparent uncoupling of the HR and disease resistance. *ndr1-1* differs from *nim1* and *npr1* in that resistance is restored upon INA treatment (K. Century and B. Staskawicz, personal communication). Therefore, the *ndr1-1* mutation probably precedes SA accumulation in the SAR signal transduction pathway, whereas *nim1* and *npr1* presumably act subsequent to SA accumulation.

CONCLUSION

When a pathogen is perceived by a host plant, a series of responses can be activated. Some of these responses, such as the synthesis and release of ethylene, may predispose the plant to further infection and thus contribute to disease susceptibility (Ecker, 1995). Other responses may contribute to the active defense of the host against the pathogen. One of these resistance responses is the SAR signal transduction pathway. Evidence that this response is important for resistance is that when the pathway is blocked through design (e.g., in NahG transgenic plants) or mutation (e.g., *nim1*, *npr1*, *ndr1*), the plant's defense is compromised. Furthermore, when the pathway is stimulated by exogenous compounds such as BTH or INA, or by mutation (e.g., *lsd*, *acd2*, *cim3*, *cpr1*), the host's defense is strengthened.

Although it is clear that the SAR signal transduction pathway is central to disease resistance, there are still many unanswered questions. What is the identity of the translocated signal? How is SA synthesized after pathogen infection? What is the receptor for SA, INA, and BTH? A detailed understanding of this pathway is important for both practical and theoretical reasons.

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REFERENCES

Alexander, D., Stinson, J., Pear, J., Glascock, C., Ward, E., Goodman, R.M., and Ryals, J. (1992). A new multigene family inducible by tobacco mosaic virus or salicylic acid in tobacco. Mol. Plant-Microbe Interact. **5**, 513–515.

Alexander, D., Glascock, C., Pear, J., Stinson, J., Ahl-Goy, P., Gut-Rella, M., Goodman, R.M., and Ryals, J. (1993a). Systemic acquired resistance in tobacco: Use of transgenic expression to study the functions of pathogenesis-related proteins. In Advances in Molecular Genetics of Plant–Microbe Interactions, Vol. 3, E.W. Nester and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 527–533.

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., and Ryals, J. (1993b). Increased tolerance to two Oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. USA **90**, 7327–7331.

Alibert, G., and Ranjeva, R. (1971). Research on the enzymes catalyzing the biosynthesis of phenolic acids in *Quercus pedunculata*. I. Formation of the first members of the cinnamic series and benzoic series. FEBS Lett. **19**, 11–14.

Antoniw, J., and White, R. (1980). The effects of aspirin and polyacrylic acid on soluble leaf proteins and resistance to virus infection in five cultivars of tobacco. Phytopathol. Z. **98**, 331–341.

Asselin, A., Grenier, J., and Cote, F. (1985). Light-influenced extracellular accumulation of b (pathogenesis-related) proteins in *Nicotiana* green tissue induced by various chemicals or prolonged floating on water. Can. J. Bot. **63**, 1276–1283.

Beffa, R., Szell, M., Meuwly, P., Pay, A., Vogeli-Lange, R., Métraux, J.-P., Meins, F., and Nagy, F. (1995). Cholera toxin elevates pathogen resistance and induces defense reactions in transgenic tobacco plants. EMBO J. **14**, 5753–5761.

Bi, Y.-M., Kenton, P., Mur, L., Darby, R., and Draper, J. (1995). Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression. Plant J. **8**, 235–245.

Boi, J.F., Linthorst, H.J.M., and Cornelissen, B.J.C. (1990). Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. **28**, 113–138.

Bowles, D.J. (1990). Defense-related proteins in higher plants. Annu. Rev. Biochem. **59**, 873–907.

Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F., and Dong, X. (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell **6**, 1845–1857.

Cameron, R.K., Dixon, R., and Lamb, C. (1994). Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. Plant J. **5**, 715–725.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6**, 1583–1592.

Century, K.S., Holub, E.B., and Staskawicz, B.J. (1995). *NDR1*, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. Proc. Natl. Acad. Sci. USA **92**, 6597–6601.

Chen, Z., and Klessig, D.F. (1991). Identification of a soluble salicylic acid-binding protein that may function in signal transduction in the plant disease-resistance response. Proc. Natl. Acad. Sci. USA **88**, 8179–8183.

Chen, Z., Silva, H., and Klessig, D.F. (1993). Involvement of reactive oxygen species in the induction of systemic acquired resistance by salicylic acid in plants. Science **242**, 883–886.

Chen, Z.X., Malamy, J., Henning, J., Conrath, U., Sanchezcasas, P., Silva, H., Ricigliano, J., and Klessig, D.F. (1995). Induction, modification, and transduction of the salicylic acid signal in plant defense responses. Proc. Natl. Acad. Sci. USA **92**, 4134–4137.

Chester, K.S. (1933). The problem of acquired physiological immunity in plants. Q. Rev. Biol. **8**, 275–324.

Cohen, Y., Gisi, U., and Niderman, T. (1993). Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic acid methyl ester. Phytopathology **83**, 1054–1062.

Dangl, J.L. (1993). Applications of *Arabidopsis thaliana* to outstanding issues in plant–pathogen interactions. Int. Rev. Cytol. **144**, 53–83.

Dangl, J.L., Dietrich, R.A., and Richberg, M.H. (1996). Death don't have no mercy: Cell death programs in plant–microbe interactions. Plant Cell **8**, 1793–1807.

Delaney, T., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266**, 1247–1250.

Delaney, T., Friedrich, L., and Ryals, J. (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl. Acad. Sci. USA **92**, 6602–6606.

Dempsey, D.M., Wobbe, K., and Klessig, D.F. (1993). Resistance and susceptible responses of *Arabidopsis thaliana* to turnip crinkle virus. Phytopathology **83**, 1021–1029.

Dietrich, R.A., Delany, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A., and Dangl, J.L. (1994). Arabidopsis mutants simulating disease resistance response. Cell **77**, 565–577.

Dixon, R.A. (1986). The phytoalexin response: Elicitation, signaling, and control of host gene expression. Biol. Rev. Camb. Philos. Soc. **61**, 239–292.

Durner, J., and Klessig, D.F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. Proc. Natl. Acad. Sci. USA **92**, 11312–11316.

Ecker, J.R. (1995). The ethylene signal transduction pathway in plants. Science **268**, 667–675.

Enyedi, A.J., Yalpani, N., Silverman, P., and Raskin, I. (1992). Localization, conjugation and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc. Natl. Acad. Sci. USA **89**, 2480–2484.

Fauth, M., Merten, A., Hahn, M.G., Jeblick, W., and Kauss, H. (1996). Competence for elicitation of H_2O_2 in hypocotyls of cucumber is induced by breaching the cuticle and is enhanced by salicylic acid. Plant Physiol. **110**, 347–354.

Friedrich, L., Vernooij, B., Gaffney, T., Morse, A., and Ryals, J. (1995). Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. Plant Mol. Biol. **29**, 959–968.

Friedrich, L., Lawton, K., Dincher, S., Winter, A., Staub, T., Uknes, S., Kessmann, H., and Ryals, J. (1996). Benzothiadiazole induces systemic acquired resistance in tobacco. Plant J. **10**, 61–70.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261**, 754–756.

Gianinazzi, S., Martin, C., and Vallée, J.C. (1970). Hypersensibilité aux virus, température et protéines solubles chez le *Nicotiana* Xanthi n.c. Apparition de nouvelles macromolécules lors de la répression de la synthèse virale. C. R. Acad. Sci. Ser. III Sci. Vie **270**, 2382–2386.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.-H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H., and Ryals, J. (1996). Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell **8**, 629–643.

Greenberg, J.T., Guo, A., Klessig, D.F., and Ausubel, F.M. (1994). Programmed cell death in plants—A pathogen-triggered response activated coordinately with multiple defense functions. Cell **77**, 551–563.

Hammond-Kosack, K.E., and Jones, J.D.G. (1996). Resistance gene-dependent plant defense responses. Plant Cell **8**, 1773–1791.

Hunt, M., and Ryals, J. (1996). Systemic acquired resistance signal transduction. Crit. Rev. Plant Sci. **15**, 583–606.

Iwata, M., Suzuki, Y., Watanabe, T., Mase, S., and Sekizawa, Y. (1980). Effect of probenzole on the activities related to the resistant reaction in rice plant. Ann. Phytopathol. Soc. Jpn. **46**, 297–306.

Jach, G., Gornhardt, B., Mundy, J., Logemann, J., Pinsdorf, P., Leah, R., Schell, J., and Maas, C. (1995). Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J. **8**, 97–109.

Kauss, H. (1987). Some aspects of calcium-dependent regulation in plant metabolism. Annu. Rev. Plant Physiol. **38**, 47–72.

Kauss, H., and Jeblick, W. (1995). Pretreatment of parsley suspension cultures with salicylic acid enhances spontaneous and elicited production of H_2O_2. Plant Physiol. **108**, 1171–1178.

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., Uknes, S., and Ryals, J. (1994). Induction of systemic acquired resistance in plants by chemicals. Annu. Rev. Phytopathol. **32,** 439–459.

Kunkel, B.N. (1996). A useful weed put to work: Genetic analysis of disease resistance in *Arabidopsis thaliana*. Trends Genet. **12,** 63–69.

Langford, A.N. (1948). Autogenous necrosis in tomatoes immune from *Cladosporium fulvum* Cooke. Can. J. Res. **26,** 35–64.

Lawton, K., Uknes, S., Friedrich, L., Gaffney, T., Alexander, D., Goodman, R., Métraux, J.-P., Kessmann, H., Ahl-Goy, P., Gut-Rella, M., Ward, E., and Ryals, J. (1993). The molecular biology of systemic aquired resistance. In Mechanisms of Defence Responses in Plants, B. Fritig and M. Legrand, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 422–432.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol. Plant-Microbe Interact. **8,** 863–870.

Lawton, K., Friedrich, L., Hunt, M., Weymann, K., Staub, T., Kessmann, H., and Ryals, J. (1996). Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. Plant J. **10,** 71–82.

León, J., Yalpani, N., Lawton, M.A., and Raskin, I. (1993a). Salicylic acid biosynthesis in healthy and virus-inoculated tobacco. In Plant Signals in Interactions with Other Organisms, J. Schultz and I. Raskin, eds (Rockville, MD: American Society of Plant Physiologists), pp. 262–265.

León, J., Yalpani, N., Raskin, I., and Lawton, M.A. (1993b). Induction of benzoic acid 2-hydroxylase in virus-inoculated tobacco. Plant Physiol. **103,** 323–328.

León, J., Lawton, M.A., and Raskin, I. (1995). Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. Plant Physiol. **108,** 1673–1678.

Levine, A., Pennell, R.I., Alvarez, M.A., Palmer, R., and Lamb, C. (1996). Calcium-mediated apoptosis in a plant hypersensitive resistance response. Curr. Biol. **6,** 427–437.

Linthorst, H.J.M. (1991). Pathogenesis-related proteins of plants. Crit. Rev. Plant Sci. **10,** 123–150.

Liu, D., Kashchandra, G., Raghothama, P., Hasegawa, P.M., and Bressan, R.A. (1994). Osmotin overexpression in potato delays development of disease symptoms. Proc. Natl. Acad. Sci. USA **91,** 1888–1892.

Low, P.S., and Merida, J.R. (1996). The oxidative burst in plant defense: Function and signal transduction. Physiol. Plant. **96,** 533–542.

Malamy, J., Carr, J.P., Klessig, D.F., and Raskin, I. (1990). Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science **250,** 1002–1004.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. **88,** 936–942.

Mauch-Mani, B., and Slusarenko, A.J. (1994). Systemic acquired resistance in *Arabidopsis thaliana* induced by prediposing infection with a pathogenic isolate of *Fusarium oxysporum*. Mol. Plant-Microbe Interact. **7,** 378–383.

Mauch-Mani, B., and Slusarenko, A.J. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of Arabidopsis to *Peronospora parasitica*. Plant Cell **8,** 203–212.

Mehdy, M.C. (1994). Active oxygen species in plant defense against pathogens. Plant Physiol. **105,** 467–472.

Métraux, J.-P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M., and Ryals, J. (1989). Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA **86,** 896–900.

Métraux, J.-P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science **250,** 1004–1006.

Métraux, J.-P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E. (1991). Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In Advances in Molecular Genetics of Plant–Microbe Interactions, Vol. 1, H. Hennecke and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 432–439.

Meyerowitz, E.M. (1992). Introduction to the *Arabidopsis* genome. In Methods in Arabidopsis Research, C. Koncz, N.-H. Chua, and J. Schell, eds (Riveredge, NJ: World Scientific Publishing), pp. 100–118.

Mölders, W., Buchala, A., and Métraux, J.-P. (1996). Transport of salicylic acid in tobacco necrosis virus-infected cucumber plants. Plant Physiol. **112,** 787–792.

Nasser, W., De Tapia, M., Kauffmann, S., Montasser-Kouhsari, S., and Burkard, G. (1988). Identification and characterization of maize pathogenesis-related proteins. Four maize PR proteins are chitinases. Plant Mol. Biol. **11,** 529–538.

Neuenschwander, U., Friedrich, L., Delaney, T., Vernooij, B., Kessmann, H., and Ryals, J. (1995a). Activation of plant disease resistance. Aspects Appl. Biol. **42,** 217–225.

Neuenschwander, U., Vernooij, B., Friedrich, L., Uknes, S., Kessmann, H., and Ryals, J. (1995b). Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? Plant J. **8,** 227–233.

Neuenschwander, U., Lawton, K., and Ryals, J. (1996). Systemic acquired resistance. In Plant–Microbe Interactions, Vol. 1, G. Stacey and N.T. Keen, eds (New York: Chapman and Hall), pp. 81–106.

Ponstein, A.S., Bres-Vloemans, S.A., Sela-Buurlage, M.B., van den Elzen, P.J.M., Melchers, L.S., and Cornelissen, B.J.C. (1994). A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. Plant Physiol. **104,** 109–118.

Rasmussen, J.B., Hammerschmidt, R., and Zook, M.N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. Plant Physiol. **97,** 1342–1347.

Redei, G.P., and Koncz, C. (1992). Classical mutagenesis. In Methods in *Arabidopsis* Research, C. Koncz, N.-H. Chua, and J. Schell, eds (Riveredge, NJ: World Scientific Publishing), pp. 17–82.

Rueffer, M., Steipe, B., and Zenk, M.H. (1995). Evidence against specific binding of salicylic acid to plant catalase. FEBS Lett. **377,** 175–180.

Ryals, J., Ward, E., and Métraux, J.-P. (1992). Systemic acquired resistance: An inducible defense mechanism in plants. In Inducible Plant Proteins: Their Biochemistry and Molecular Biology, J.L. Wray, ed (Cambridge, UK: Cambridge University Press), pp. 205–229.

Sandoz, L. (1991). Plant pathogenesis-related proteins. International application published in the Patent Cooperation Treaty, No. WO 92/20800.

Schlumbaum, A., Mauch, F., Vogeli, U., and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. Nature **324,** 365–367.

Schnitzler, J.-P., Madlung, I., Rose, A., and Seitz, H.U. (1992). Biosynthesis of *p*-hydroxybenzoic acid in elicitor-treated carrot cell cultures. Planta **188,** 594–600.

Shirasu, K., Dixon, R.A., and Lamb, C. (1996). Signal transduction in plant immunity. Curr. Opin. Immunol. **8,** in press.

Shulaev, V., León, J., and Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell **7,** 1691–1701.

Summermatter, K., Sticher, L., and Métraux, J.-P. (1995). Systemic responses in *Arabidopsis thaliana* infected and challenged with *Pseudomonas syringae* pv. *syringae*. Plant Physiol. **108,** 1379–1385.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in Arabidopsis. Plant Cell **4,** 645–656.

Uknes, S., Winter, A., Delaney, T., Vernooij, B., Friedrich, L., Morse, A., Potter, S., Ward, E., and Ryals, J. (1993). Biological induction of systemic acquired resistance in Arabidopsis. Mol. Plant-Microbe Interact. **6,** 692–698.

Vance, C.P., Kirk, T.K., and Sherwood, R.T. (1980). Lignification as a mechanism of disease resistance. Annu. Rev. Phytopathol. **18,** 259–288.

Van Loon, L.C. (1985). Pathogenesis-related proteins. Plant Mol. Biol. **4,** 111–116.

Van Loon, L.C., and Van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN' II. Changes in protein constitution after infection with tobacco mosaic virus. Virology **40,** 199–211.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6,** 959–965.

Vernooij, B., Friedrich, L., Ahl-Goy, P., Staub, T., Kessmann, H., and Ryals, J. (1995). 2,6-Dichloroisonicotinic acid–induced resistance to pathogens does not require the accumulation of salicylic acid. Mol. Plant-Microbe Interact. **8,** 228–234.

Walbot, V., Hoisington, D.A., and Neuffer, M.G. (1983). Disease lesion mimics in maize. In Genetic Engineering of Plants, Vol. 3, T. Kosuge, C. Meredith, and A. Hollaender, eds (New York: Plenum Publishing), pp. 431–442.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3,** 1085–1094.

Weymann, K., Hunt, M., Uknes, S., Neuenschwander, U., Lawton, K., Steiner, H.-Y., and Ryals, J. (1995). Suppression and restoration of lesion formation in Arabidopsis *lsd* mutants. Plant Cell **7,** 2013–2022.

Whalen, M.C., Innes, R.W., Bent, A.F., and Staskawicz, B.J. (1991). Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell **3,** 49–59.

White, R.F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology **99,** 410–412.

White, R.F., Rybicki, E.P., Von Wechmar, M.B., Dekker, J.L., and Bol, J.F. (1987). Detection of PR-1 type proteins in Amaranthaceae, Chenopodiaceae, Gramineae and Solanaceae by immunoblotting. J. Gen. Virol. **68,** 2043–2048.

Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, M., van den Elzen, P.J.M., and Cornelissen, B.J.C. (1991). Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell **3,** 619–628.

Wolter, M., Hollricher, K., Salamini, F., and Schulze-Lefert, P. (1993). The *mlo* resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. Mol. Gen. Genet. **239,** 122–128.

Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A., and Raskin, I. (1991). Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell **3,** 809–818.

Yalpani, N., León, J., Lawton, M., and Raskin, I. (1993a). Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. Plant Physiol. **103,** 315–321.

Yalpani, N., Shulaev, V., and Raskin, I. (1993b). Endogenous salicylic acid levels correlate with accumulation of pathogenesis-related proteins and virus resistance in tobacco. Phytopathology **83,** 702–708.

Yazaki, K., Heide, L., and Tabata, M. (1991). Formation of *p*-hydroxybenzoic acid from *p*-coumaric acid by cell free extract of *Lithospermum erythrorhizon* cell cultures. Phytochemistry **7,** 2233–2236.

Zhu, Q., Maher, E.A., Masoud, S., Dixon, R.A., and Lamb, C.J. (1994). Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio/Technology **12,** 807–812.